

BETAINE ANALOGUES AND RELATED COMPOUNDS FOR BIOMEDICAL APPLICATIONS

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ABSTRACT

Living cells accumulate compensatory solutes for protection against the harmful effects of extreme environmental conditions such as high salinity, temperature and desiccation. Even at high concentrations these solutes do not disrupt the normal cellular functions and at times counteract by stabilizing the cellular components. These properties of compensatory solutes have been exploited for stabilizing proteins and cells *in vitro*. Betaines are widespread natural compensatory solutes that have also been used in other applications such as therapeutic agents and polymerase chain reaction (PCR) enhancers. Some biomedical applications of novel synthetic analogues of natural betaines were investigated.

Natural compensatory solutes are either dipolar zwitterionic compounds or polyhydroxyl compounds, and the physical basis of compensation may differ between these, so one focus was on synthetic betaines with hydroxyl substituents. The majority of the synthetic solutes stabilized different model proteins against stress factors such as high and low temperatures. The presence of hydroxyl groups improved protection against desiccation. The observed stabilization effect is not just on the catalytic activity of the enzyme, but also on its structural conformation. Synthetic compensatory solutes have a potential application as protein stabilizers.

Dimethylthetin was evaluated as a therapeutic agent and found to be harmful in a sheep model. However, from the study we were able to generate a large-animal continuous ambulatory peritoneal dialysis (CAPD) model and showed that glycine betaine could be added to the dialysis fluid in chronic renal failure.

Some synthetic compensatory solutes reduce the melting temperatures of DNA better than most natural solutes. Synthetic solutes were identified that have potential to enhance PCR and could replace some reagents marketed by commercial suppliers.

Density, viscosity and molecular model data on the solutes showed correlations with the biochemical effects of the solutes, but no physical measurements were found that reliably predicted their potential for biotechnological applications.

Chapter 1 - INTRODUCTION

1.1 GENERAL INTRODUCTION

Living organisms survive in a wide variety of environmental conditions, including extremes of salinity, temperature and humidity. These environmental stresses disrupt key properties of the cells such as enzymatic catalysis, binding of ligands by enzymes, protein subunit assembly and protein solubility (Yancey et al., 1982). Organisms have developed a number of adaptive strategies to cope with these environmental stresses and one of these is the accumulation of high concentrations of low molecular weight solutes in the intracellular fluid. These solutes, termed 'osmolytes', are used as osmotic agents to maintain the osmotic balance in the cell and are found both in prokaryotic and eukaryotic cells (Yancey et al., 1982). Commonly occurring osmolytes are classified as sugars, polyhydric alcohols, amino acids and their derivatives, and methylamines (Somero, 1992). As these organic osmolytes are accumulated to extremely high concentrations without perturbing the cellular functions, they are also called 'compatible solutes' (Brown and Simpson, 1972). The accumulation of compatible solutes in cells is a general mechanism for protein stabilization in the face of high intracellular osmolarity and is simpler than evolution of many intracellular proteins.

Urea is an organic osmolyte which is found in different animals along with other osmolytes. The presence of urea is difficult to explain as it is a strong perturbant of macromolecules and is commonly used to denature proteins. Urea is found in certain cartilaginous fish (Yancey and Somero, 1978) and also in the inner medulla of mammalian kidneys (Yancey, 1992). Wherever there are potentially harmful concentrations of urea, there also appears to be the co-occurrence of second type of organic osmolyte, typically a methylamine. Methylamines such as glycine betaine, trimethylamine-*N*-oxide (TMAO) and sarcosine are known to counteract the perturbation of proteins by urea (Yancey and Somero, 1978). Therefore, these osmolytes were further termed as 'counteracting solutes'.

In a study by Bedford et al. (1995), it was shown that the medullary and papillary regions of the kidney of domesticated red deer were rich in betaine, sorbitol and free amino acids, suggesting their role as osmolytes. NMR and HPLC analyses of the organic constituents of tissues of elephant fish revealed that glycine betaine and a compatible osmolyte, taurine, are present in high concentrations. These results suggested that osmolytes are accumulated to counteract the external osmotic stress (Bedford et al., 1997).

Apart from their counteracting effect, a few solutes are also known to stabilize the cellular components and are referred to as ‘compensatory solutes’ (Galinski et al., 1997; Gilles, 1997). Compensatory solutes can be classified into two main categories of neutral polar organic compounds. The first group includes polyols or polyhydric alcohols (e.g. trehalose and sorbitol) and the second group includes zwitterions containing a relatively hydrophobic cationic region (e.g. glycine betaine and ectoine). Compensatory solutes are known to affect the structuring of water in solutions (Galinski et al., 1997). Solute that increase the structure of water are termed ‘kosmotropes’ and those which decrease the structure of water are termed ‘chaotropes’.

As described above, these organic solutes are often referred with different terms. Other than simply referring to them as solvent additives, compounds which are able to stabilize cells against osmotic stress and help in maintaining osmotic equilibrium are termed ‘osmolytes’. Solute which do not disturb the cellular mechanism even when present in very high concentration are called ‘compatible solutes’ and the solutes that are known to stabilize certain cell components are termed ‘counteracting’ or ‘compensatory’ solutes. Hereafter in this thesis, these solutes will be referred to as ‘compensatory solutes’ (natural or synthetic).

Compensatory solutes, due to their effective stabilization of cell components are of great interest to biotechnologists. Till now, biotechnologists have focused primarily on natural compensatory solutes such as trehalose and glycine betaine. However, solutes such as trehalose are expensive to produce or purchase. Little attention has been paid to finding inexpensive alternatives to these natural stabilizers. Only a few systematic studies have

tried to examine the effect of analogues of natural solutes. Lloyd et al. (1992; 1994) studied a few simple analogues of natural kosmotropes and showed that relationships existed between the solute structure and its effectiveness to stabilize proteins. Synthetic betaines have been shown to counter the effects of high NaCl and urea concentrations using cultures of *E. coli* as a model system (Randall et al., 1996). Recently, a synthetic compatible solute, homoectoine, was shown to be a potent PCR enhancer (Schnoor et al., 2004). Hence, designing new synthetic compensatory solutes may help biotechnologists explore novel and inexpensive ways to stabilize proteins.

The native folded structure of a protein is sensitive to changes in environmental conditions such as temperature, pressure, moisture content, and presence of salts and other solutes. Significant perturbations of these conditions can cause changes in secondary and tertiary structure of the protein, leading to a partial or complete loss of their activity. Different ways of stabilizing proteins for long-term storage or for use at high and low temperatures have been investigated in the past. Consequently, stabilization of proteins and its mechanism have been an interesting area of research for the last three decades. Several mechanisms of protein stabilization have been proposed; the preferential exclusion model by Timasheff and co-workers (Gekko and Timasheff, 1981), the osmophobic effect proposed by Bolen and co-workers (Bolen and Baskakov, 2001) and protection of proteins against low temperatures (e.g. freeze-drying) by Carpenter and Crowe (1989). A review of these proposed mechanisms is detailed in Chapter 2 of this thesis.

The majority of the studies carried out to explore the mechanism of protein stabilization have used only a few naturally occurring solutes. These proposed mechanisms have not been able to explain why some of the solutes are protein specific and stabilize some proteins, but fail to stabilize others. In many cases the use of these natural solutes is insufficient to stabilize proteins completely. To gain a greater understanding of these stabilization mechanisms it is proposed to synthesize compensatory solutes which are structurally slightly different, and to study their stabilization effect on different proteins. This approach may give new insight into the mechanism of protein stabilization and also

provide biotechnologists with alternate inexpensive solutes for the purpose of protein stabilization.

Glycine betaine (GB) is possibly the most widely distributed compensatory solute in nature. It enables growth under conditions of cell stress in bacteria (Redulier et al., 1984; Chambers and Kunin, 1985; Randall et al., 1996), higher plants (Rhodes and Hanson, 1993) and mammals (Burg, 1992). GB also stabilizes biological macromolecules against heat denaturation (Santoro et al., 1992), freezing (Lloyd et al., 1994) and urea (Yancey et al., 1982).

Other than being a compensatory solute, glycine betaine plays an important role in nature acting as a methyl donor in homocysteine metabolism. Homocysteine (Hcy) is an intermediate in the metabolism of the essential amino acid methionine. In a pathway catalyzed by betaine-homocysteine methyltransferase (BHMT- E.C. 2.1.1.5), glycine betaine donates a methyl group to homocysteine forming methionine and *N,N*-dimethylglycine (Figure 1.1).

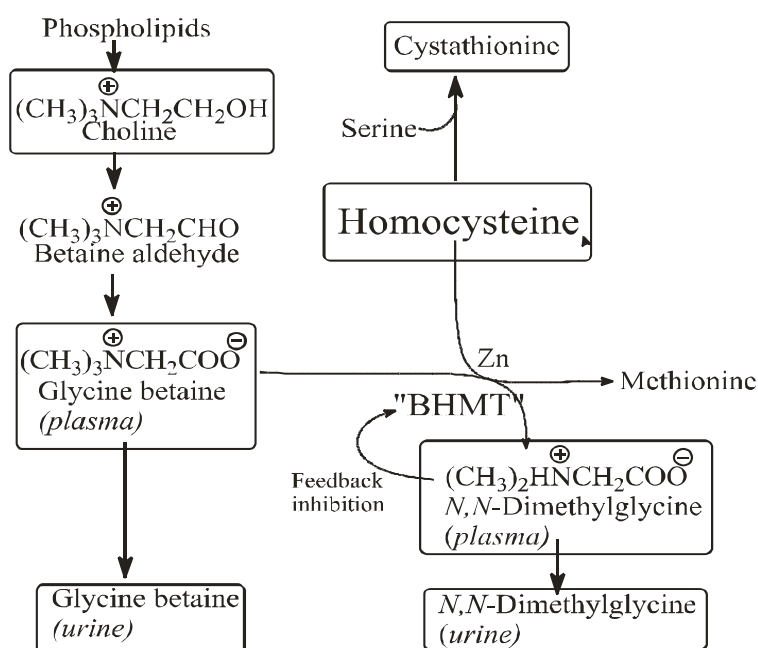
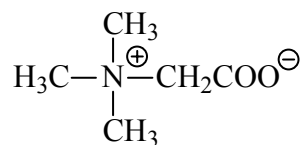


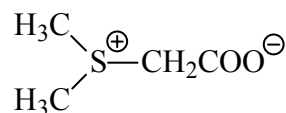
Figure 1.1: Homocysteine metabolism

It has been shown that 80% of chronic renal failure (CRF) patients receiving dialysis treatment have elevated plasma Hcy (Bostom and Lathrop, 1997; Soria et al., 1990; Hultberg et al., 1993), which in turn has been linked to increased risk of vascular disease (cardio, cerebral and peripheral) (Boushey et al., 1995; Nygard et al., 1997; Welch and Loscalzo, 1998). The cause(s) of elevated levels of Hcy in renal failure patients is unclear. However, the kidney primarily metabolizes Hcy rather than excreting it, and thus it is likely that the higher levels of plasma Hcy in CRF patients is a result of disturbed Hcy metabolism. Oral supplementation with GB in humans has proven useful for lowering plasma Hcy concentrations (Wilcken et al., 1983; McGregor et al., 2002; Olthof et al., 2003), however, long-term compliance is problematic and diarrhoea has been reported during therapy (Knopman and Patterson, 2001). Consequently, it would be useful to explore new and effective ways of treating renal failure patients with GB to increase the methyl donor availability.

One of the products of BHMT pathway, *N,N*-dimethylglycine, is a potent feedback inhibitor of BHMT (McKeever et al., 1991; Millan & Garrow, 1998), and this may be one of the causes for disturbed Hcy metabolism leading to elevated Hcy concentrations. Dimethylthetin (DMT), a synthetic analogue of GB, may be a potential therapeutic agent for lowering plasma Hcy. Purified BHMT has been shown to utilize DMT as a substrate at thirty-times the rate of GB (Goeger & Ganther, 1993). Furthermore, the demethylated product of DMT, methylthioacetate, does not inhibit BHMT as does *N,N*-dimethylglycine (Garrow, 1997), and it has been shown that a single dose of DMT is more effective for lowering Hcy in rats when compared to an equivalent dose of GB (Slow et al., 2004), thus suggesting that DMT could be used as a therapeutic homocysteine-lowering agent.



Glycine betaine [GB]



Dimethylthetin [DMT]

Higher concentrations of glycine betaine have also been shown to reduce the DNA melting temperatures by eliminating the base pair composition dependence of DNA (Rees et al., 1993). Glycine betaine destabilizes the double helical conformation of DNA and the melting temperature decreases approximately linearly with increasing GB concentrations. It has been shown that addition of glycine betaine in a polymerase chain reaction (PCR) assay enhances/improves the amplification of GC-rich DNA sequences (Henke et al., 1997).

1.2 OBJECTIVES

Based on these various applications of glycine betaine, a natural compensatory solutes, we decided to synthesize a range of analogues of glycine betaine and its related compounds, and investigate their effectiveness in various biomedical applications. The objectives of this thesis were:

- To synthesize a range of novel compensatory solutes.
- To explore the application of synthetic compensatory solutes as protein stabilizers. For this, we plan to study the protective effects of synthetic solutes on different model enzymes against the deleterious effects of high and low temperatures.
- To explore the medical application of betaine analogues by studying GB and DMT treatment for reducing plasma Hcy levels in an animal model of acute renal failure.
- To investigate the effects of synthetic compensatory solutes on DNA melting temperatures and to identify new PCR enhancers for amplification of GC-rich DNA sequences.
- To gather more physical data for novel synthetic compensatory solutes and to attempt to provide more information towards the understanding of mechanisms of protein stabilization.

1.3 THESIS OVERVIEW

Based on the objectives, this thesis has been divided into following chapters:

Chapter 2: This chapter contains a review of literature on the proposed mechanisms of protein stabilization by co-solvents/compensatory solutes.

Chapter 3: This chapter describes the methods of syntheses of the various compensatory solutes used in this thesis. Results of ^1H NMR and elemental analyses of the synthesized solutes are presented.

Chapter 4: Stabilization of different model enzymes by synthetic compensatory solutes against heat denaturation, freeze-drying and freeze-thawing is examined in this chapter. The ability of compensatory solutes to prevent the unfolding of chymotrypsin is also examined.

Chapter 5: This chapter examines the effect of addition of DMT and GB to dialysis fluid in order to reduce the plasma Hcy levels in a sheep model of acute renal failure.

Chapter 6: The effect of synthetic compensatory solutes on melting temperatures of three different DNA samples is examined in this chapter. This chapter also investigates the effectiveness of a few synthetic solutes to act as PCR enhancers.

Chapter 7: Density and viscosity measurements and calculation of apparent hydration numbers of various compensatory solutes are presented and discussed in this chapter. Results of theoretical calculations of interactions of compensatory solutes with water are also presented.

Chapter 8: This chapter contains the discussion and conclusions of various applications of synthetic compensatory solutes explored in this thesis. Recommendations for future work are also detailed.

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Chapter 2 - Proteins and Stabilization Mechanisms

2.1 PROTEINS

Proteins, meaning “of prime importance” in Greek, are fundamental constituents of all cells and tissues in our body. They are essential for the function and structure of all living cells and viruses. Proteins are large molecules which are made up of 20 different smaller molecules called amino acids, an organic acid that contains an amino group. Though amino acids are classified into different groups, the common feature is that they all have central carbon atom attached to a hydrogen atom, an amino group and a carboxylic group.

Several amino acids are joined together by peptide bonds to form a polypeptide. A peptide bond is an amide linkage between the carboxyl group of one amino acid and the amino group of another amino acid, with the splitting out of a molecule of water. Proteins may be considered as complex polypeptides with one or more polypeptide chains in each molecule. Peptide bonds joining to form an unbranched polypeptide chain are considered the primary structure of a protein. In secondary structure, several polypeptide chains are joined together and held in definite folded shapes by hydrogen bonds, a bonding that usually occurs between the carbonyl and amide groups of the peptide chain. The tertiary structure of a protein is formed by several types of bonds between the polypeptide chains that hold the structure in a more complex and a rigid shape.

Primarily, proteins are classified into two broad categories, globular proteins and fibrous proteins. Fibrous proteins are insoluble in water and are used for structural purposes in organisms (e.g. skeletal muscles). Globular proteins are tightly folded, soluble in water and have specific functions (e.g. enzymes, hormones and antibodies). Enzymes are one of the major groups of globular proteins and are considered as organic catalysts that accelerate many reactions in a living cell.

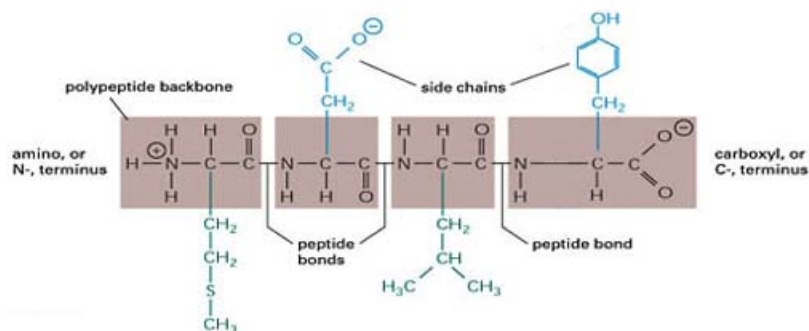


Figure 2.1: The peptide bonds, peptide backbone and side chains of a polypeptide. (<http://folding.stanford.edu/education/protein.html>)

A more appropriate definition of an enzyme is: *Enzymes are proteins, formed by a living cell, which catalyze a thermodynamically possible reaction by lowering the activation energy so the rate of reaction is compatible with the conditions in the cell* (Routh et al., 1973). Enzymes are classified based on the type of chemical reaction they catalyze. The six major classes of enzymes are,

1. *Oxidoreductases*, enzymes involved in physiological oxidation processes.
2. *Transferases*, enzymes catalyzing the transfer of a chemical group from one substrate to another.
3. *Hydrolases*, enzymes which catalyze hydrolytic reactions.
4. *Lyases*, enzymes catalyzing the removal of chemical groups without hydrolysis.
5. *Isomerases*, enzymes which catalyze isomerization reactions.
6. *Ligases*, enzymes catalyzing the linking together of two molecules with the breaking of a bond.

To be biologically active, proteins and enzymes need to maintain their native conformational structure. Changes in temperature, pH and environmental conditions, lead to unfolding (denaturation) of protein structure, which changes their chemical properties and render them inactive. *In vivo*, living cells have developed their own strategies, like accumulation of certain solutes, to stabilize proteins against environmental changes.

Organisms maintain their biological activity in the face of severe denaturing stresses such as heat, dehydration and extreme solution conditions like high salt and urea (Somero, 1986; Yancey et al., 1982). These organisms protect themselves against the denaturing stresses by producing tens to hundreds of millimolar intracellular concentrations of certain low molecular weight compounds, known as osmolytes (Yancey, 1982). Osmolytes have been shown to provide stability to enzymes usually without affecting their catalytic activity (Bowlus and Somero, 1979; Borowitzka and Brown, 1974). Researchers have explored different methods to stabilize proteins for their long-term storage and use. Proteins usually unfold and become inactive during their storage in solution or when subjected to low temperatures during freezing. In order to avoid unfolding and to stabilize proteins, co-solvents may be added to the protein solution.

2.2 MECHANISMS OF PROTEIN STABILIZATION BY COSOLVENTS

2.2.1 Preferential Exclusion, Preferential Hydration and Preferential Interaction

It was known for many years that addition of certain substances to aqueous solution of proteins results in stabilization of their native conformation. Some of the most commonly used compounds for this purpose were sucrose, glycerol and ethylene glycol. In 1976, Timasheff and colleagues tried to investigate, at the molecular level, the mechanism by which certain solvent systems stabilize the structure of biological macromolecules. Timasheff et al. (1976) reported that protein structure stabilizing solvents are preferentially excluded from the domain of the protein. In their study of α -chymotrypsin in 4 M glycerol they showed such exclusion existed and proposed that this exclusion is thermodynamically unfavorable and the system will tend to reduce it by decreasing the area of solvent-protein contact through enhancement of the protein self-association.

To understand this mechanism further, Gekko and Timasheff (1981) made measurements of preferential interactions of proteins with components in a water-glycerol solvent system using six different proteins. They showed that all six proteins used were preferentially hydrated in the water-glycerol solvent system. Preferential binding data

indicated that the chemical potential of a protein (or its activity coefficient) increases with increasing glycerol concentration. Since glycerol is known to repel non-polar substances quite effectively and interacts favorably with water, the presence of glycerol in the aqueous medium should increase the hydrophobicity of the protein. Thus, the non-polar/hydrophobic groups would be expected to react unfavorably to contact with the mixed solvent and would prefer to migrate into the interior of the protein, out of contact with the solvent. However, such a migration is not possible due to the tight packing of the three-dimensional structure of the protein and also due to the fact that hydrophobic groups are covalently linked to the polypeptide chain of the protein. As a result, they proposed, the converse takes place, i.e. the water and glycerol molecules redistribute themselves in the vicinity of the protein molecule.

When protein denatures or unfolds, there is an increase in the surface of contact between protein and solvent, and it also exposes additional hydrophobic residues. Hence, unfolding in the presence of glycerol would increase the thermodynamically unfavorable situation and require the use of more free energy for unfolding than in water. They concluded that the presence of glycerol should tend to favour the more folded or native state. The main drawback of this study was that all the measurements of preferential interactions were done on the native protein and not on the denatured or unfolded protein. Furthermore, they generalized for all proteins based on the effect of glycerol then observed on six model proteins.

Continuing the effort to understand the stabilization of globular proteins by preferential hydration and the mechanism of co-solvent interactions with proteins, Arakawa et al. (1990a), undertook a study to examine the effect of MgCl_2 on protein stability at acid pH and at salt concentrations of 0.5-1.5 M, conditions at which MgCl_2 induced preferential hydration of proteins. They included 2-methyl-2,4-pentanediol (MPD) in their study as it had been shown that the presence of MPD induced strong preferential hydration of ribonuclease A (Pittz and Timasheff, 1978), and also due to the prediction that MPD should be a protein denaturant (Pittz and Bello, 1971). Their analysis of the relations between preferential interactions and structural stabilization of proteins brought out the

reasons why preferential interactions of native proteins with solvent additives do not necessarily correlate with protein stability.

From their results, Arakawa et al. (1990a) classified co-solvents into two categories. In their first category, consisting of good stabilizers, like sucrose and MgSO_4 , the protein-solvent interactions were determined mainly by the properties of the solvent. As illustrated in their figure (Figure 2.2A) the interaction is always that of preferential hydration, whether the protein is native or unfolded, and the principal mechanism of interaction is the strong increase in the surface free energy (surface tension) of water upon their addition. During unfolding, due to the increase in surface of protein-solvent contact and the surface free energy, the equilibrium shifts toward the native compact state resulting in structure stabilization. In their second category, as illustrated in Figure 2.2B, it is the chemical nature of the protein surface that determines the interactions (repulsive or attractive). In this category, the preferential interactions varied strongly with the pH of the system, concentration of solvent additive and the state of folding of the protein. This category contained MPD (repelled by charges, bound to non-polar regions), MgCl_2 (excluded by surface tension effect, attracted by Mg^{2+} binding to non-polar regions) and polyethylene glycol (excluded by steric effect, bound to non-polar regions).

Arakawa et al. (1990a) concluded, as illustrated in Figure 2.2, that when the protein-solvent interactions are independent of the chemical nature of the protein surface, the effect will always be that of stabilization and if the interactions are mainly due to the chemical nature of the protein surface, addition of co-solvent may either stabilize or destabilize the protein. They also concluded that the measurement of the interactions for just the native protein does not give direct insight into the effect of the solvent on protein stability.

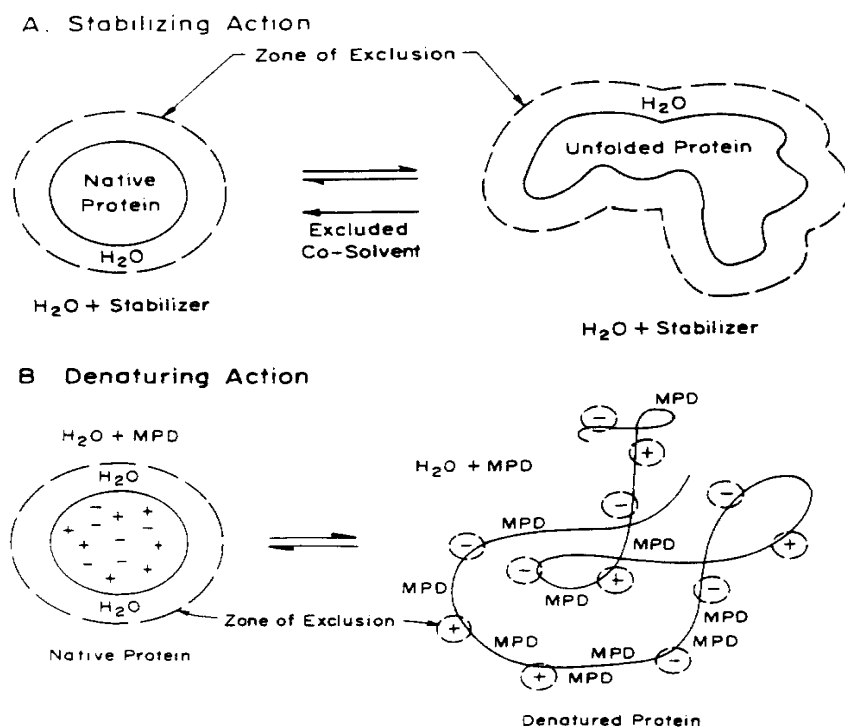
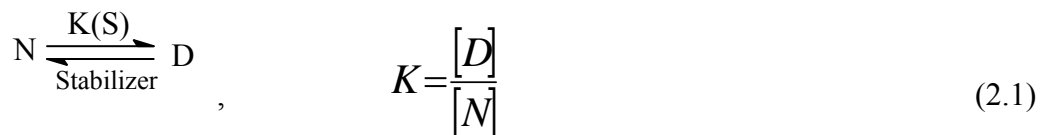


Figure 2.2: Protein-Solvent interactions (A) Protein denaturation reaction in the presence of a stabilizing co-solvent. (B) In the denaturation reaction, shown for water-MPD system, MPD binding to solvent-exposed non-polar regions in the denatured state. (Arakawa *et al.*, 1990a)

The thermodynamic principles that govern the structure stabilization by solvent components were explained by Timasheff and Arakawa (1997). They considered the destabilization reaction, that is, protein denaturation, and expressed it as simple two-state equilibrium between the native 'N' and denatured (unfolded or destabilized) 'D' states. If by the addition of solvent additive S, the equilibrium be shifted toward the native form of the protein, then the reaction can be expressed by:



Thus, by definition, the equilibrium constant of the above reaction is a function of the concentration of the stabilizer S.

The Wyman linkage relation (Wyman, 1964), expresses quantitatively the effect of the stabilizer on the equilibrium constant. This relation states that, when the co-solvent concentration changes, the change in the equilibrium constant is given by the difference in the number of co-solvent molecules bound by the denatured and native protein molecules:

$$\frac{d \log K}{d \log a_s} = \Delta \nu_s = \nu^D - \nu^N \quad (2.2)$$

where a_s is the thermodynamic activity of the additive, S, related to its free concentration by the activity coefficient; $\Delta \nu_s$ is the difference between the number of moles of solution component S (stabilizer) bound per mole of protein between the denatured (ν^D) and native (ν^N) states of the protein.

From equation (2.2), Timasheff and Arakawa (1997) stated that in the case of stabilization the reaction will be shifted to the left, so the equilibrium constant must decrease with an increase in concentration of the additive, thus, $(d \log K/d \log a_s)$ will be negative, so $\Delta \nu_s$ will also be negative. In other words, there must be less binding of the stabilizer to the denatured form of the protein than to the native one. To measure these, they used a dialysis equilibrium technique to measure the binding of stabilizing solvents to a variety of proteins in the native state. The results of their studies showed a negative number of moles of additive per mole of protein. Figure 2.3 shows the interpretation of the results of their dialysis equilibrium experiment. In Figure 2.3 (A) we see that at the end of the experiment, the solution inside the bag has a higher concentration of the additive than the bulk solvent, and in Figure 2.3 (B), we see the opposite, that is, the solution inside the bag has lower concentration of the additive than the bulk solvent.

From the dialysis experiment, it was found that sugars and amino acids were preferentially excluded from the protein surface, thus stabilizing the proteins. Interestingly, all the co-solvents that stabilized the protein also raised the surface tension of water. These observations suggested that the stabilization of proteins by co-solvents is

due to the increase of the surface tension in their presence. This was consistent with the classic results of Gibbs (1878), who showed that substances that lower the surface tension of water accumulate at the surface and those that raise surface tension are depleted from the surface. The analyses of interfacial tension at the protein surface contained assumptions about the protein-solvent interface.

Surface tension, classically, is measured at a flat homogeneous water-air interface, which varies with temperature and solvent composition. However, the protein surface is not flat and the effect of its curvature on the interface had to be accounted for. Also, the surface of the protein is not chemically homogeneous and can be regarded as a mixture with varying polarity, hydrophobicity and electro static charge. The increase of surface tension of water by several molecules gave a poor correlation between the experimentally measured preferential interactions and the values expected from the surface tension measurements, like ArgHCl and LysHCl with bovine serum albumin (Kita et al., 1994) and for MgCl₂ (Arakawa et al., 1990a). Because ArgHCl and LysHCl have the potential to interact directly with the proteins by hydrogen bonding to peptide groups, Kita et al. (1994) proposed that the measured preferential interactions are a summation of the effects of the increase in surface tension and weak binding.

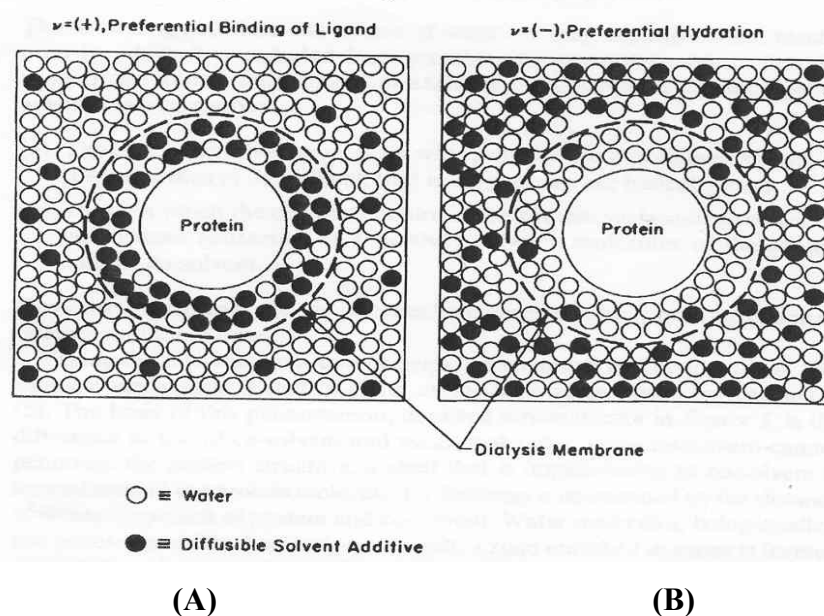


Figure 2.3: Solvent component distribution at dialysis equilibrium (A) Preferential binding (B) Preferential hydration. (Timasheff and Arakawa, 1997)

Timasheff and co-workers laid the foundation for understanding the mechanism of protein stabilization by co-solvents. However, there were limitations in their studies. They considered only a few co-solvents for their studies, and their proposed mechanisms were mainly based on the effect of co-solvent on the native protein not on its denatured form. Their mechanism emphasized the interaction of co-solvents with protein side-chains and does not mention effects on the peptide backbone, which also contributes to the hydrophobic effect of proteins.

2.2.2 Steric exclusion mechanism

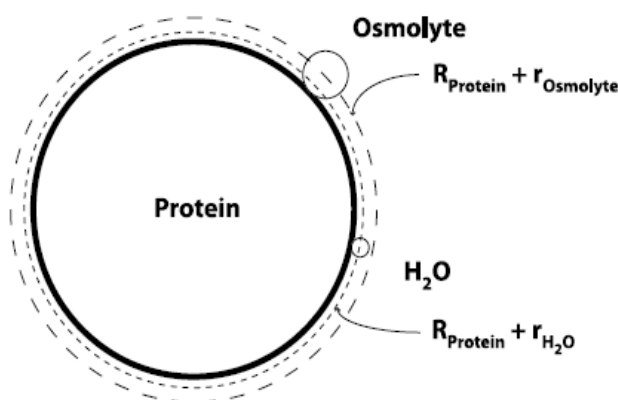


Figure 2.4: The steric exclusion mechanism of preferential hydration. Water, protein and osmolyte (co-solvent) are represented by spherical geometry (*Bolen, 2004*).

Kauzmann (1949) (quoted in Schachmann and Lauffer, 1949) first proposed the steric exclusion mechanism to explain the preferential hydration of co-solvents. The difference in the size of co-solvent and water molecules is the basis of this phenomenon and according to it, if the co-solvent cannot penetrate the protein structure, a shell that is impenetrable to co-solvent is formed around the protein molecule. The thickness of this shell is determined by the distance of closest approach of protein and co-solvent. However, water molecules, which are smaller in size, can penetrate within this shell. This results in a zone around the protein molecule that is enriched in water. Effectively, this is preferential hydration and preferential exclusion of the co-solvent. This hypothesis has

been studied by Bhat and Timasheff (1992) using several proteins, with varying molecular weight and polarity, and with both high and low molecular weight polyethylene glycol (PEG) molecules. They concluded that steric exclusion is the principal source of preferential hydration of proteins in the presence of polyethylene glycols.

Steric exclusion does not apply to all cases. Urea and guanidine hydrochloride have radii larger than that of water and preferential interaction measurements on proteins using these solutes have shown that these strong denaturants do not preferentially hydrate proteins and moreover, they bind to the native protein molecules (Arakawa and Timasheff, 1984; Timasheff, 1992). These two denaturants show that solutes that have preferences of favorably interacting with the chemical groups on the protein surface can override the excluded volume effects that cause preferential hydration. Thus, affinity and steric effects both contribute to the distribution of solute and water at the protein surface (Bolen, 2004).

2.2.3 Osmophobic Effect

Bolen and co-workers have tried to explore the mechanism of protein stabilization with their studies on osmolytes and related compounds. They studied the effects of osmolytes that are chemically similar to one another, choosing glycine based osmolytes and studying their stabilizing effect on ribonuclease A (RNase A) and hen egg white lysozyme (HEW lysozyme) against thermal unfolding (Santoro et al., 1992). They observed that T_m , the midpoint temperature of thermal unfolding transition for a protein, increased for both RNase A and HEW lysozyme in the presence of osmolytes. These osmolytes seemed to stabilize proteins even well beyond the physiological concentration and moreover, the degree of stabilization was extraordinary. They also showed that RNase A will refold in a completely reversible manner in the presence of up to 8 M sarcosine, which indicated that basic rules for RNase A refolding did not change as a function of the concentration of the osmolytes. Some of their results also suggested that there is a tendency for these osmolytes to destabilize the proteins at very high

concentration. They concluded that osmolytes should be able to stabilize any enzyme or protein to which it is exposed and it does not matter whether the protein evolved in the presence of osmolytes or not.

Baskakov and Bolen (1998) studied the effects of two osmolytes, urea (a denaturant) and trimethylamine-*N*-oxide (TMAO) (an effective stabilizer). They showed that urea is no better at solubilizing hydrophobic side chains than is TMAO, as the overall side chain interactions with urea and TMAO are favorable, which implies that both these solutes should denature proteins. They suggested that the magnitude of the favorable vs. unfavorable interactions of the protein backbone with the solute, primarily determines whether the solute will be a denaturing or a protecting osmolytes. Qu et al. (1998) raised the possibility that the backbone might play a role in protein folding in water and also whether solvophobicity of the backbone in water had been overlooked so far as a contributor to the folding of protein in aqueous solution.

Bolen and Baskakov (2001) wrote a comprehensive review of their studies with osmolytes and proteins, and their proposed mechanism. They classified the unfavorable interactions between a solvent component (an osmolytes) and a protein functional group (peptide backbone) as solvophobic and called the unfavorable interaction the ‘osmophobic effect’. They argued that experimental measurements made by Timasheff and co-workers could not distinguish a solvophobic mechanism from other mechanisms. By means of their measurements of transfer Gibbs energy of amino acid side-chains and peptide backbone from water to a variety of naturally occurring osmolytes, Bolen and co-workers presented the evidence that the mechanism of osmolytes action is solvophobic and proposed that osmophobic effect is a thermodynamic force of biological importance in protein folding.

They revealed the consequences of the preferential exclusion of osmolytes from native and denatured proteins species using a thermodynamic cycle (Figure 2.5). In the thermodynamic cycle, reactions 2 and 4 represent the transfer of denatured (D) and native protein (N) from water to a fixed concentration of osmolyte. They argued that, compared

to the native state, denatured protein exposes more protein fabric to solvent, thus resulting in denatured state of the protein being more solvophobic toward osmolyte than the native state, making $\Delta G_{D[Os]}$ a significantly more positive quantity than $\Delta G_{N[Os]}$.

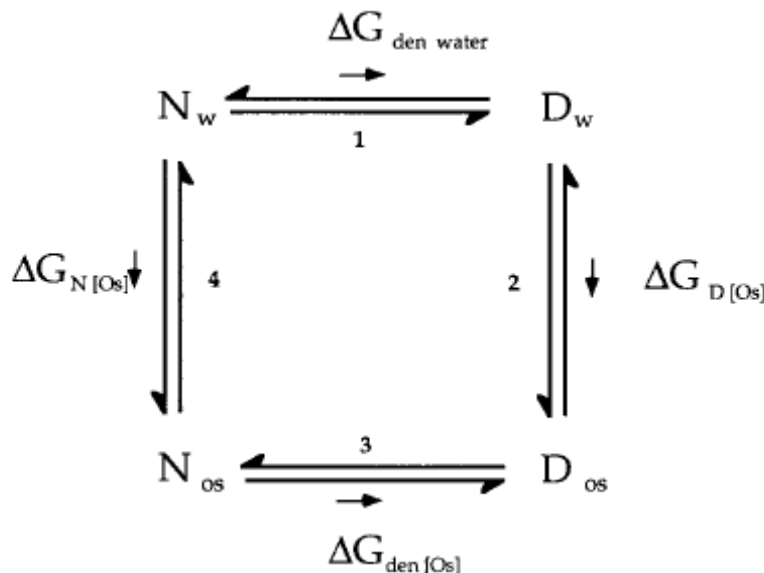


Figure 2.5: Thermodynamic cycle of protein denaturation (*Bolen and Baskakov, 2001*).

According to them, the thermodynamic cycle requires that $\Delta G_{D[Os]} - \Delta G_{N[Os]} = \Delta G_{den[Os]} - \Delta G_{den\ water}$, and this condition forces the conclusion that denaturation of protein ($\Delta G_{den[Os]}$) is less favorable (more positive quantity) in the presence of osmolyte than it is in water ($\Delta G_{den\ water}$). Because osmolytes raise the Gibbs energy of the denatured state far more than they do the native state, proteins are more stable in the presence of osmolytes than they are in water.

They obtained transfer Gibbs energy measurements of amino acid side-chains and backbone, from water to various osmolyte solutions, in order to determine the propensities of the side-chains and backbone to interact with several of naturally occurring osmolytes. They estimated the transfer Gibbs energy of native and denatured protein, $\Delta G_{N[Os]}$ and $\Delta G_{D[Os]}$, using transfer Gibbs energy data and models of denatured protein developed by Creamer et al. (1997).

The results of their measurements showed that the peptide backbone is responsible for the unfavorable interaction with osmolyte, and that the side chains collectively favour interaction with osmolyte (Liu and Bolen, 1995; Wang and Bolen, 1997; Qu et al., 1998). As suggested by Timasheff and co-workers, the origin of the hydrophobic effect in proteins lies in the unfavorable interaction between apolar side-chains and water. Similarly, Bolen and co-workers proposed that the origin of the hydrophobic effect in proteins lies in the unfavorable interaction between the peptide backbone and an osmolyte. The concept of both these forces is similar because an unfavorable interaction between a structural component of the protein and a component of solution is responsible for the force involved in protein folding.

Bolen and co-workers also suggested that the interactions of protecting osmolytes and urea (denaturant) with protein side-chains are of less importance during denaturation than their respective favorable and unfavorable interactions with the peptide backbone (Bolen and Baskakov, 2001). The principal difference between urea and protecting osmolytes is due to the fact that urea interaction with peptide backbone is favorable and dominant over its favorable interactions with side-chains, while the protecting osmolytes interact unfavorably with backbone but favorably with side-chains (Wang and Bolen, 1997). They concluded that osmolytes (through the osmophobic effect) focus on the peptide backbone, a part of protein separate from side-chains, for protein folding in dilute aqueous medium (Bolen and Baskakov, 2001).

2.2.4 Protein Stabilization at Low Temperatures (Freeze-thawing and Freeze-drying)

Freeze-thawing and freeze-drying are the two methods commonly used for long-term storage of proteins and during transportation of proteins. For storage, some proteins are frozen and then thawed before use. During freeze-thawing and freeze-drying, proteins have to endure stresses such as low temperatures and formation of ice, which destabilize them. It has been found that many proteins are not stable against these stresses (Carpenter et al., 1986; Carpenter et al., 1987; Carpenter and Crowe, 1988a&b). A number of compounds have been examined for their effects on retention of enzyme activity upon

freeze-thawing and freeze-drying (Carpenter and Crowe, 1988b). During freeze-thawing, compounds that stabilize proteins in their solution state, protect proteins against low temperatures, and those compounds that are known to destabilize or denature proteins in solution, enhance damage due to freeze-thawing. On the other hand, it has been found that only sugars protect proteins from damage due to desiccation (Carpenter et al., 1987; Carpenter and Crowe, 1988a; Carpenter and Crowe, 1989). In the sections below, the effect of freeze-thawing and freeze-drying on proteins has been explained further.

2.2.4.1 Freeze-thawing of proteins

For long-term storage, proteins are often frozen and then thawed before use. During this process, proteins are exposed to critical stresses such as low temperature and formation of ice. Cold denaturation has been accounted for the inactivation of many proteins during freeze-thawing (Becktel and Schellman, 1987; Privalov, 1990). During freezing, protein molecules are excluded from the ice crystals and are subjected to chemical and physical changes that occur in the non-ice phase. With the formation of ice, the concentration of all solutes increases and if the solutes present are destabilizing, then the concentrating effect can lead to protein denaturation (von Hippel et al., 1969). Also, if the protein solution is made up of a buffer such as sodium phosphate, then during freezing the solution can undergo a dramatic decrease in the pH, resulting in destabilization of the protein (Chilson et al., 1965; van den Berg and Rose, 1969). Thus, the combination of factors arising during freeze-thawing, low temperatures and high destabilizing salt concentration, can damage the protein. The duration of exposure of a protein to these conditions can also influence the degree of damage. This has been proved by the finding that the loss of enzyme activity during freezing and thawing is often inversely correlated with the cooling and warming rates (Chilson et al, 1965; Whittam and Rosano, 1973).

The perturbations that have been shown to destabilize the protein can be protein-specific. However, it is widely agreed that any factor that alters protein stability in non-frozen aqueous solution will tend to have the same qualitative effect during freeze-thawing (Arakawa et al., 2001). Also, it has been shown that increasing the protein concentration

will increase the stability of the protein during freeze-thawing (Carpenter and Crowe, 1988b). To prevent the damage that occurs to the protein during freeze-thawing, a number of cryoprotectants have been employed. The most common cryoprotectants are sugars, polyols, synthetic polymers and amino acids (Carpenter and Crowe, 1988b).

Several mechanisms have been proposed to explain the effect of these cryoprotectants on proteins. The only mechanism that seems to be applicable to most of the known compounds is the preferential exclusion mechanism proposed by Timasheff and co-workers described in Section 2.2.1 of this chapter. Most of these compounds have been shown to be preferentially excluded from the surface of protein, and the compounds like urea and guanidine-HCl, which are known to preferentially bind to the proteins, have been found to cause additional damage during freeze-thawing (Carpenter and Crowe, 1988b; Arakawa et al., 1990b). Arakawa et al. (2001) proposed that the destabilizing conditions that may arise during freeze-thawing, such as concentration of solutes and alterations in solution pH, can be viewed simply as other types of perturbation induced in solution. Thus, the basic thermodynamic principles governing protein stability in the frozen state is not different from those observed in non-frozen aqueous systems (Arakawa et al., 2001).

However, there is a group of compounds, whose effect on proteins in non-frozen aqueous system, do not correlate with their effect during freeze-thawing (e.g. PEG and MPD), as these compounds destabilize proteins at slightly higher temperatures (Section 2.2.1). In fact, PEG has been found to be a very effective cryoprotectant (Carpenter and Crowe, 1988b). This kind of effect has been attributed to the temperature dependence of hydrophobic interactions between proteins and co-solvents. At low temperatures, these solutes are preferentially excluded and the hydrophobic interactions are weaker, whereas at relatively higher temperatures ($>25^{\circ}\text{C}$), the hydrophobic interactions become stronger and it leads to preferential binding (Arakawa et al., 1990b).

2.2.4.2 Freeze-drying of proteins

Freeze-drying or lyophilization is a method used in preparation of protein products, when the proteins are not sufficiently stable for long-term storage as aqueous solutions. The process of lyophilization consists of freezing the protein solution followed by drying in vacuum. It is known that the second step of lyophilization, that is drying, takes place in two phases: primary drying, which removes frozen water through sublimation, and secondary drying which removes non-frozen 'bound' water (Arakawa et al., 2001). Freeze-drying often destabilizes proteins due to the conformational instability of many proteins when subjected to freezing and subsequent dehydration stresses (Crowe et al., 1990). In contrast to free-thawing, where freezing is the only stress, freeze-drying poses two stresses, freezing and drying. Thus, to protect proteins against freeze-drying, both these fundamentally different stresses have to be overcome.

Several additives have been tried in order to protect proteins against freeze-drying. However, the solutes that provide stabilization are sugars and polyols. The mechanism by which these solutes provide protection is not completely understood. Clearly, co-solute stabilization during freeze-drying is more complex than that of cryoprotection (Crowe et al., 1990). Many effective cryoprotectants fail to stabilize proteins during dehydration. It has been suggested that the mechanism of solute-induced stabilization during dehydration is fundamentally different from that for proteins in aqueous or frozen systems (Carpenter and Crowe, 1989). They have also suggested that the thermodynamic principles that explain protein stabilization by preferential exclusion are not applicable for freeze-drying as the solvent itself is removed from the system.

Carpenter and Crowe (1989), in their study of effect of carbohydrates during freeze-drying of proteins, have suggested that these solutes protect proteins because they hydrogen-bond to the dried protein acting as a water substitute, when the hydration shell of protein is removed. They used Fourier transform infrared spectroscopy (FTIR) to study the interactions between dried proteins and carbohydrates. They proved that hydrogen bonding does occur between proteins and carbohydrates during drying, suggesting that

such bonding may be a requisite for labile proteins to be preserved during drying. Their results are illustrated in Figure 2.6.

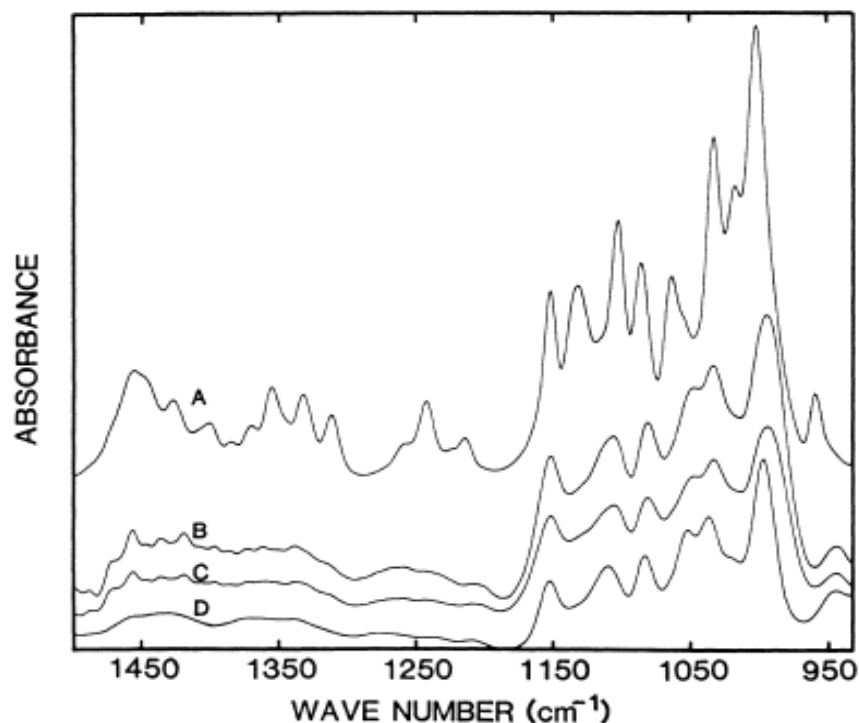


Figure 2.6: Infrared spectra. (Spectrum A) Trehalose freeze-dried alone. (Spectrum B) Freeze-dried trehalose + 0.3 g of lysozyme/g of trehalose. (Spectrum C) Freeze-dried trehalose + 0.1 g of BSA/g of trehalose. (Spectrum D) Hydrated trehalose. (*Arakawa et al., 2001*)

Figure 2.6 illustrates the results of trehalose and protein interactions in the dried state. From the figure it is evident that the spectra for trehalose dried in the presence of either lysozyme or bovine serum albumin (BSA) are remarkably similar to that for hydrated trehalose, but very different from the spectrum of crystalline trehalose. Thus, they concluded that proteins form hydrogen bonds with the polar groups in the sugar, serving the same role for dried trehalose as water does for hydrated trehalose.

Based on the results that proteins serve as water substitutes for dried carbohydrates, Carpenter and Crowe (1989) stated that the converse must also be true, which is

carbohydrates serve as water substitutes for dried proteins. To test this hypothesis, they investigated the influence of trehalose on the infrared spectrum of lysozyme. The results are illustrated in Figure 2.7. The effectiveness of trehalose is evident by the similarity of peaks at 1580, 1540 and 1520 cm^{-1} .

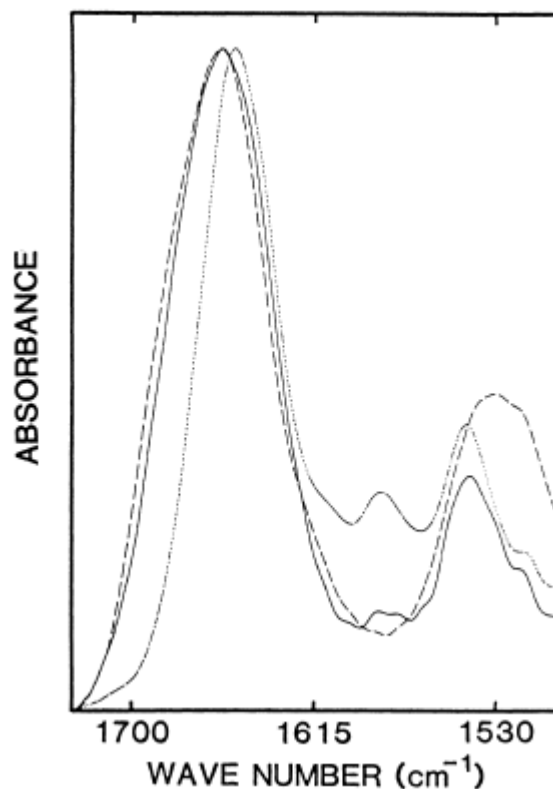


Figure 2.7: Amide band region for hydrated lysozyme (dotted line), lysozyme freeze-dried alone (dashed line), and lysozyme freeze-dried in the presence of trehalose (solid line) (Arakawa *et al.*, 2001).

Based on these results, it was proposed that hydrogen-bonding of the sugar to the protein is mandatory for the sugar to preserve dried proteins. This conclusion has been supported by results of Lippert and Galinski (1992), who showed that stabilization of freeze-dried PFK and lactate dehydrogenase (LDH) by compatible solutes, ectoines, depended on the presence of hydroxyl groups on the molecule.

Other investigators have proposed a different mechanism for protection of proteins by carbohydrates during freeze-drying. Franks et al. (1991) proposed that the glass formation in the dried state is responsible for stabilization, which means that the immobilization of the protein and additives in the glassy state results in the protection of the protein from chemical and conformational degradation. Though this is a good hypothesis, there is evidence which argue against it. In a study by Tanaka et al. (1991), they examined the ability of various saccharide monomers and oligomers to protect catalase during freeze-drying. It was observed that the ability of the saccharides to protect the protein decreased with increase in the length of the chain. However, results from Franks et al. (1991) show that with the increase in the length of the chain, saccharides become better glass-formers as they undergo glass transitions at higher temperatures. These results are exactly opposite to the proposed glass formation mechanism.

Of the proposed mechanisms of protection of proteins against freeze-drying, the effect of saccharides, serving as water substitutes for dried proteins, seems to be the most effective explanation. However, the mechanism by which this interaction between protein and the sugar occurs is not completely understood yet.

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Chapter 3 - SYNTHESIS OF COMPENSATORY SOLUTES

3.1 INTRODUCTION

Naturally occurring compensatory solutes such as glycine betaine, TMAO, trehalose and sorbitol (Figure 3.1), have been studied with great interest by biotechnologists due to their ability to stabilize proteins against different stresses and their effectiveness in stabilizing cell components. Moreover, to understand the mechanism of protein stabilization, many researchers have used these natural solutes. Some of the natural solutes, like trehalose, are very expensive to purchase and there are only a limited number of alternatives available in the present day market.

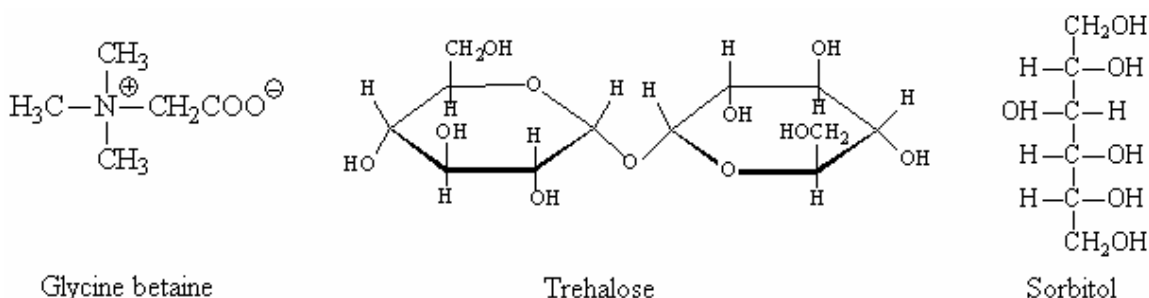


Figure 3.1: Structures of some natural compensatory solutes.

There has been less effort to synthesize inexpensive analogues of these natural solutes. A few researchers have tried to synthesize analogues of compensatory solutes and have studied their effect on macromolecules. Lloyd et al. (1992, 1994) investigated some simple analogues of natural kosmotropes for their effectiveness as cryoprotective additives. Synthetic betaines like, propio betaine (*N, N, N*-trimethyl-*N*-(2-carboxyethyl) ammonium, inner salt) and dimethylthetin (*N,N*-dimethyl-*N*-carboxymethyl sulfonium, inner salt), have been shown to counter the effects of high NaCl and urea concentrations in *E. coli* cultures (Randall et al., 1996).

A class of synthetic betaines, called sulfobetaines, has been investigated for their properties as protein solubilizing agents. These sulfobetaines are betaines containing a

sulfonate functional group instead of a carboxylate. Vuillard et al. (1995c) synthesized zwitterionic non-detergent sulfobetaines (NDSB) and showed that NDSBs are non-denaturing additives that can significantly enhance protein extraction yields and do not interfere with the binding of proteins to ion exchange chromatography columns. In a study by the same group (Vuillard et al., 1995a), they observed that overnight incubation of β -galactosidase in NDSB solutions resulted in an increase in enzymatic activity. NDSBs were also shown to have the capacity to stabilize halophilic proteins against deactivation in low salt concentrations (Vuillard et al., 1995b).

Synthetic compensatory solutes have the ability to open new insights into the mechanism of protein stabilization, as they could help us understand the relation between the structure of solutes and their stabilization effect on proteins. Lloyd et al. (1992; 1994) showed patterns of relationship between the solute structure and their effectiveness as cryoprotective additives. Results obtained in a membrane-protein extraction show that NDSBs with C₃ bridge between N and S were more efficient than NDSBs with C₄ bridge (Vuillard et al., 1995b). Compensatory solutes with hydroxyl group in their structure have been observed to have increased stabilization effect against different stresses (Goeller and Galinski, 1999; Randall et al., 1996).

One of the most widely occurring natural compensatory solutes, glycine betaine, has applications in biological and medical fields. Apart from acting as an osmolyte, glycine betaine is a good protein stabilizer. In the laboratory it has been used in polymerase chain reaction (PCR) as an enhancer (Frackman et al., 1998) due to its ability to reduce DNA melting temperatures (Rees et al., 1993). It is also used a therapeutic agent for treatment of hyperhomocysteinemia, a condition which causes vascular diseases in renal failure patients (Knopman and Patterson, 2001; Olthof et al., 2003). This application as a therapeutic agent does not depend on its properties as a compatible solute, but on its other biochemical role as a methyl group donor.

These applications of compensatory solutes, and results indicating the potential applications of synthetic solutes inspired the syntheses of a number of inexpensive novel

compensatory solutes in our laboratory. A range of analogues of glycine betaine and its related compounds were synthesized in order to study their effectiveness in different biological and medical applications. Furthermore, these synthetic solutes may help to get a better understanding of the mechanism of protein stabilization.

In this chapter, the syntheses of different compensatory solutes will be presented, along with their characterization by ^1H NMR and elemental analysis.

3.2 MATERIALS AND METHODS

Trimethylamine (40 wt% in water), acrylic acid, dimethylaminoethanol (deanol), bromoacetic acid, *N*-methyldiethanolamine, triethanolamine, 3-dimethylamino-1-propanol, 3-(dimethylamino)-1,2-propanediol, 25% w/v solution of vinylsulfonic acid sodium salt in water (VSA), 1,3-propane sultone, 1, 4-butane sultone, 1-methylpiperidine and 1-methylpyrrolidine were purchased from Aldrich, USA. *N*-Methylmorpholine and dimethylsulfide were purchased from Sigma, USA. ^1H NMR spectra were obtained on a Varian Unity 300 MHz instrument. Elemental analyses were carried out at Campbell Microanalytical Laboratory, University of Otago, Dunedin.

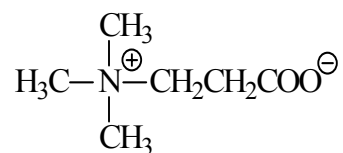
The majority of the compensatory solutes described in this chapter were obtained as clean white crystals. They were characterised by elemental analysis and their structures confirmed by ^1H NMR.

3.2.1 Ion-exchange chromatography

Solutes that were obtained in the hydrohalide form were converted to the free base by passing them through a Dowex 50 column (H^+ form). The length and diameter of the column used depended on the number of moles of the solute to be treated. The column was prepared by first batch washing the resin with $\sim 4\text{ M}$ HCl and then washing it with large amounts of distilled water until the washings were neutral. The crude hydrohalide form of the solute was dissolved in a small amount of water and poured onto the column

and the column was then washed with large amounts of water. The betaine is retained by the column. Starting materials (if present) and halide ions which do not have affinity for the column are eluted. The positively charged betaines were eluted from the column by washing with ~ 5 M aqueous ammonia solution. The solution obtained was evaporated under reduced pressure at ~ 30 $^{\circ}\text{C}$ in a rotary evaporator. The absence of halide ions in the eluant and in the final product was confirmed by testing samples with AgNO_3 solution.

3.2.2 Propio betaine [PB]



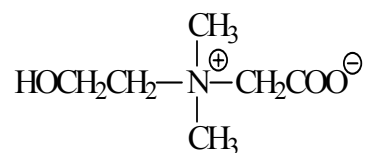
N, N, N-Trimethyl-N-(2-carboxyethyl) ammonium, inner salt

CAS # 6458-06-6

Propiobetaine was prepared by mixing trimethylamine (40 wt% in water) and acrylic acid in a molar ratio of 1.5:1. The mixture was allowed to stand for 3 days at room temperature. The product, a viscous liquid, was dissolved in warm isopropanol and a large volume of acetone was added. The solid precipitated was then filtered and sucked dry. As it was deliquescent, it was recrystallised from water/acetonitrile mixtures using a rotary evaporator. A clean white powder was obtained with an approximate 80% yield.

^1H NMR (D_2O , 500 MHz) δ 2.60 (2 H, m, CH_2), 3.03 (9 H, s, $(\text{CH}_3)_3$), 3.49 (2 H, t, J 7.8 Hz, CH_2).

3.2.3 Deanol betaine [DB]



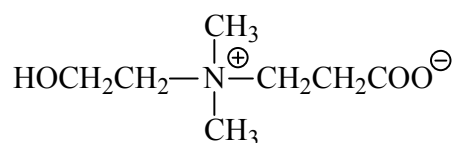
N, N-Dimethyl-*N*-(2-hydroxyethyl)-*N*-carboxymethyl ammonium, inner salt

CAS # 7002-65-5

Bromoacetic acid (0.2 moles), and dimethylaminoethanol (deanol), (0.3 moles), were dissolved in 60 mL of dichloromethane and the mixture was left to stand for 14 days at room temperature. A thick white paste of crude hydrobromide was obtained. This hydrobromide was converted to the betaine by ion exchange on a Dowex column as described in section 3.2.1. The final product was isolated from the eluant by evaporation under reduced pressure using a rotary evaporator with an approximate yield of 85 %.

¹H NMR (D₂O, 500 MHz) δ 3.19 (6 H, s, (CH₃)₂), 3.65 (2 H, m, CH₂), 3.87 (2 H, s, CH₂), 3.92 (2 H, m, CH₂).

3.2.4 Homodeanol betaine [HDB]



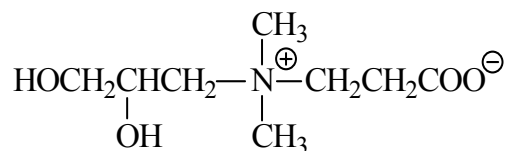
N, N-Dimethyl-*N*-(2-hydroxyethyl)-*N*-(2-carboxyethyl) ammonium, inner salt

CAS # 6249-53-2

Homodeanol betaine was prepared by mixing equimolar amounts of acrylic acid and 2-*N,N*-dimethylaminoethanol (deanol) and allowing the mixture to stand for 48 hours. The product was purified by washing several times sequentially with *n*-propanol and isopropanol, to remove the starting materials. The purified product was recrystallized from water/isopropanol mixtures. The final product yield was 80 % (approx.).

¹H NMR (D₂O, 500 MHz) δ 2.60 (2 H, t, *J* 7.8 Hz, CH₂), 3.05 (6 H, s, (CH₃)₂), 3.40 (2 H, t, *J* 4.9 Hz, CH₂), 3.55 (2 H, t, *J* 7.3 Hz, CH₂), 3.95 (2 H, m, CH₂).

3.2.5 Homoglycerol betaine [HGB]



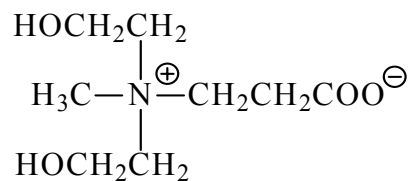
N, N-Dimethyl-N-(2,3-dihydroxypropyl)-N-(2-carboxyethyl) ammonium, inner salt

CAS # None

Acrylic acid (0.1 moles), and 3-(dimethylamino)propane-1,2-diol (0.1 moles), were mixed in 75 mL of dichloromethane. The mixture was allowed to stand at room temperature for four days. During this time the mixture separated into two layers, an upper crystalline layer and a lower liquid layer. The crystals were separated by filtration and washed sequentially with dichloromethane, isopropanol and *n*-propanol, to remove any starting materials in the product. A clean white crystalline final product was obtained with an approximate yield of 85 %.

¹H NMR (D₂O, 500 MHz) δ 2.61 (2 H, m, CH₂), 3.08 (6 H, d, *J* 5.3 Hz, (CH₃)₂), 3.35 (2 H, m, CH₂), 3.48 (2 H, m, CH₂), 3.58 (2 H, m, CH₂), 4.18 (1 H, m, CH).

3.2.6 Diethanol homobetaine [DEHB]



N,N-Diethanol-N-methyl-N-(2-carboxyethyl)ammonium, inner salt

CAS # 43192-67-2

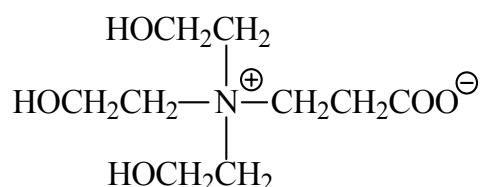
N-methyldiethanolamine (0.2 moles), and acrylic acid (0.2 moles), were mixed together in 250 mL of dichloromethane and left to stand at room temperature for 3 weeks. After day 10, the solution had started turning cloudy and small crystals commenced to form in the medium. On day 22, the reaction was worked up. The crystals were filtered off and washed with dichloromethane, isopropanol and acetone, to get rid of all the impurities

and starting materials. The final product was dried in a desiccator for 24 hours. The approximate yield was 30 %.

^1H NMR (D_2O , 500 MHz) δ 2.62 (2 H, t, J 7.8 Hz, CH_2), 3.10 (3 H, s, CH_3), 3.50 (4 H, m, $(\text{CH}_2)_2$), 3.63 (2 H, t, J 7.3 Hz, CH_2), 3.93 (4 H, m, $(\text{CH}_2)_2$).

The yield of the product by above method was very low but other methods tried led to polymer formation. Addition of hydroquinone to the reaction mixture to inhibit polymerization gave better yields. The final product was brownish in colour and not suitable for spectrophotometric analysis.

3.2.7 Triethanol homobetaine [TEHB]



N,N,N-Triethanol-*N*-(2-carboxyethyl)ammonium, inner salt

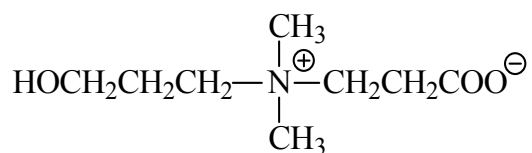
CAS # 857163-31-6

0.25 g of hydroquinone, 0.2 moles of triethanolamine and 0.2 moles acrylic acid, were added to 200 mL of dichloromethane. The solution went cloudy after mixing and separated into two phases. The mixture was left to stand at room temperature for 30 days. During this time small crystals had started depositing on the walls of the flask, and the upper layer had become quite pink. The bottom liquid layer was discarded and the upper sludgy layer was dissolved in 150 mL of warm isopropanol. Crystals were precipitated from this by adding 500 ml of cold acetone with stirring. These were filtered off and dried in a desiccator. The final product was brownish in colour and the approximate yield was 40 %.

^1H NMR (D_2O , 500 MHz) δ 2.60 (2 H, t, J 7.8 Hz, CH_2), 3.38 (2 H, t, J 5.4 Hz, CH_2), 3.56 (4 H, t, J 5.4 Hz, $(\text{CH}_2)_2$), 3.66 (2 H, t, J 7.8 Hz, CH_2), 3.86 (2 H, t, J 4.9 Hz, CH_2), 3.94 (4 H, m, $(\text{CH}_2)_2$).

As the final product was brownish in colour and not suitable for spectrophotometric analysis, attempts were made to synthesize the solute without using hydroquinone. However this always resulted in polymerized products.

3.2.8 Hydroxypropyl homobetaine [HPHB]



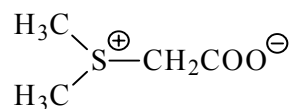
N,N-Dimethyl-*N*-(3-hydroxypropyl)-*N*-(2-carboxyethyl)ammonium, inner salt

CAS # None

3-dimethylamino-1-propanol (0.2 moles), and acrylic acid (0.2 moles), were mixed with 125 mL of dichloromethane and left to stand at room temperature. The mixture formed two phases after 4 days and by the end of 7 days, some solid material had started to appear. After 11 days the solid material was filtered off and washed several times with dichloromethane and isopropanol. The product was a sticky pale yellow deliquescent solid. This was then resuspended twice in isopropanol and filtered. The final product was dried in a desiccator. The approximate yield was 20 %.

^1H NMR (D_2O , 500 MHz) δ 1.95 (2 H, m, CH_2), 2.60 (2 H, t, J 7.8 Hz, CH_2), 3.01 (6 H, s, $(\text{CH}_3)_2$), 3.33 (2 H, m, CH_2), 3.50 (2 H, t, J 7.8 Hz, CH_2), 3.62 (2 H, t, J 5.8 Hz, CH_2).

3.2.9 Dimethylthetin [DMT]



N,N-Dimethyl-*N*-carboxymethyl sulfonium, inner salt

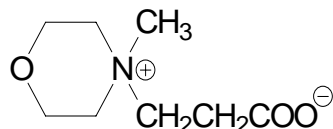
CAS # 4727-41-7

Dimethylthetin was synthesized by dissolving 0.05 moles of chloroacetic acid in 0.2 moles of dimethylsulfide. The mixture was allowed to stand for 10 days and the crystals that formed were collected by filtration. These were then washed with dichloromethane. The crude hydrochloride was converted to free base as described in section 3.2.1. The final product was isolated from the eluant by evaporation under reduced pressure using a rotary evaporator. The yield was approximately 90 %.

^1H NMR (D_2O , 500 MHz) δ 2.80 (6 H, s, $(\text{CH}_3)_2$), 4.03 (2 H, s, CH_2).

3.2.10 Cyclic Betaines

3.2.10.1 CB-1

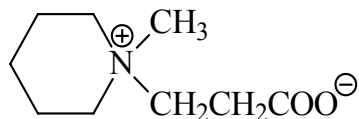


N-Methyl-*N*-(2-carboxyethyl)morpholinium, inner salt

CAS # None

Acrylic acid (0.2 moles), and *N*-methylmorpholine (0.2 moles), were mixed in 40 mL of dichloromethane and allowed to stand at room temperature. There was no apparent change in the mixture after 7 days, so the cover was taken off to allow the dichloromethane to evaporate. By the end of day 9, needle-like crystals had formed. These were filtered off and washed several times with dichloromethane and isopropanol. The final product was obtained with an approximate yield of 90 %.

^1H NMR (D_2O , 500 MHz) δ 2.62 (2 H, t, J 7.8 Hz, CH_2), 3.09 (3 H, s, CH_3), 3.42 (4 H, m, $(\text{CH}_2)_2$), 3.632 (2 H, t, J 7.8 Hz, CH_2), 3.96 (4 H, s, $(\text{CH}_2)_2$).

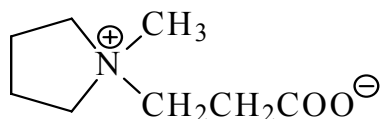
3.2.10.2 CB-2

N-Methyl-N-(2-carboxyethyl)piperidinium, inner salt

CAS # 81239-43-2

Acrylic acid (0.1 moles) and 1-methylpiperidine (0.1 moles), were mixed in 50 mL of dichloromethane. The mixture was covered and allowed to stand at room temperature. There was no change after 5 days, so the cover was taken off to allow the dichloromethane to evaporate. By the end of 10 days, the mixture had formed a hard solid layer. This was broken up and the solids were suspended in isopropanol. Further washing with *n*-propanol gave the final betaine in approximately 50% yield.

^1H NMR (D₂O, 500 MHz) δ 1.58 (2 H, m, CH₂), 1.80 (4 H, t, *J* 5.4 Hz, (CH₂)₂), 2.72 (2 H, t, *J* 7.8 Hz, CH₂), 2.95 (3 H, s, CH₃), 3.27 (4 H, m, (CH₂)₂), 3.53 (2 H, t, *J* 7.8 Hz, CH₂).

3.2.10.3 CB-3

N-Methyl-N-(2-carboxyethyl)pyrrolidinium, inner salt

CAS # 201931-52-4

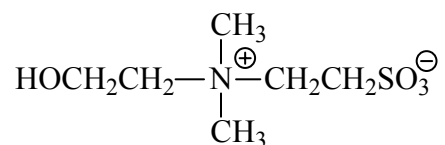
Acrylic acid (0.1 moles) and 1-methylpyrrolidine (0.1 moles) were mixed in 50 mL dichloromethane. There was a strong exothermic reaction. The mixture was covered and

allowed to stand at room temperature. Crystals started appearing after 3 days and by day 10, there were sufficient crystals in the flask for the mixture to be worked up. The crystals obtained were yellowish in colour, so they were suspended several times in isopropanol and filtered. Finally the product was washed in n-propanol. Approximate yield was 75 %. Elemental analysis and ^1H NMR were performed to confirm the structure and composition of the product.

^1H NMR (D_2O , 500 MHz) δ 2.13 (4 H, s, $(\text{CH}_2)_2$), 2.75 (2 H, t, J 7.3 Hz, CH_2), 2.95 (3 H, s, CH_3), 3.44 (4 H, m, $(\text{CH}_2)_2$), 3.54 (2 H, t, J 7.3 Hz, CH_2).

3.2.11 Sulfobetaines

3.2.11.1 SB-1



N,N-Dimethyl-*N*-(2-hydroxyethyl)-*N*-(2-sulfoethyl)ammonium, inner salt

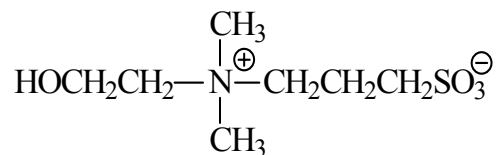
CAS # 91673-91-5

SB-1 was prepared using the method described by Barnhurst (1961). Concentrated hydrochloric acid was added to 0.05 moles of a 25% w/v solution of vinylsulfonic acid sodium salt in water. To this 0.1 moles of dimethylaminoethanol was added and the mixture was mixed well. The clear straw-coloured solution was boiled for 30 minutes, during which time the volume decreased to 60 % of that of the original solution. The mixture was allowed to cool down and evaporate further. After 3 days, 50 mL of cold conc. hydrochloric acid was added. Betaines are soluble in this medium, while the inorganic salts, which are slightly soluble, were not and were filtered off. The filtrate was concentrated to a thick syrupy consistency in a rotary evaporator at 50°C. Addition of ethanol caused the betaine to precipitate. The product obtained was further purified by

washing with dichloromethane, isopropanol and *n*-propanol. The final approximate yield was 65 %.

^1H NMR (D_2O , 500 MHz) δ 3.13 (6 H, s, $(\text{CH}_3)_2$), 3.37 (2 H, m, CH_2), 3.46 (2 H, m, CH_2), 3.71 (2 H, m, CH_2), 3.98 (2 H, m, CH_2).

3.2.11.2 SB-2



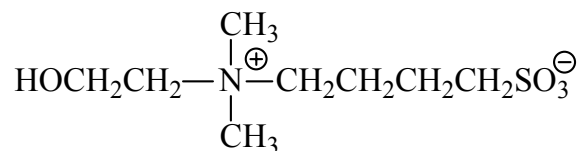
N,N-Dimethyl-*N*-(2-hydroxyethyl)-*N*-(3-sulfopropyl)ammonium, inner salt

CAS # 38880-58-9

Dimethylaminoethanol (0.1 moles), was dissolved in 125 mL of dichloromethane and 1,3-propane sultone (0.1 moles), was added and mixed well. Crystals started appearing within 15 minutes of mixing. The solution was covered and was allowed to stand for 2 days at room temperature and gave a paste. More dichloromethane was added and the solid was filtered off. The solids were resuspended in isopropanol a couple of times and finally filtered and sucked dry. The final product after drying in a desiccator was a white powder. The approximate yield was 85 %.

^1H NMR (D_2O , 500 MHz) δ 2.16 (2 H, m, CH_2) 2.89 (2 H, t, J 7.3 Hz, CH_2), 3.09 (6 H, s, $(\text{CH}_3)_2$), 3.44 (2 H, t, J 4.4 Hz, CH_2), 3.65 (2 H, m, CH_2), 3.96 (2 H, m, CH_2).

3.2.11.3 SB-3



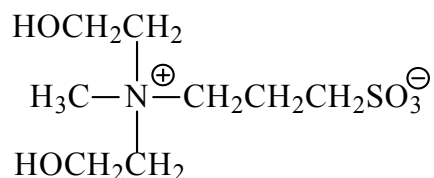
N,N-Dimethyl-*N*-(2-hydroxyethyl)-*N*-(4-sulfobutyl)ammonium, inner salt

CAS # 40820-99-3

Dimethylaminoethanol (0.1 moles) was dissolved in 20 mL of dichloromethane and to this 0.1 moles of 1,4-butane sultone was added and mixed well. The solution was covered and was allowed to stand for 3 days at room temperature. A paste formed. More dichloromethane was added and the solids isolated by filtration. The solids were washed in dichloromethane two times, resuspended in isopropanol once and finally filtered and sucked dry. Drying in a desiccator gave the final product in the form of a white powder in approximately 80 % yield.

^1H NMR (D_2O , 500 MHz) δ 1.72 (2 H, m, CH_2), 1.88 (2 H, m, CH_2), 2.89 (2 H, t, J 7.8 Hz, CH_2) 3.07 (6 H, s, $(\text{CH}_3)_2$), 3.35 (2 H, m, CH_2), 3.42 (2 H, m, CH_2), 3.96 (2 H, m, CH_2).

3.2.11.4 SB-4



N,N-Dihydroxyethyl-*N*-methyl-*N*-(3-sulfopropyl)ammonium, inner salt

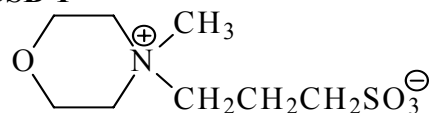
CAS # 94159-69-0

N-methyldiethanolamine (0.15 moles) and 1,3-propane sultone (0.15 moles) were mixed together in 200 mL of dichloromethane. Small crystals started forming within two hours. The mixture was allowed to stand for 3 days, after which time the crystals were filtered off and sucked dry. These were washed several times with dichloromethane and *n*-propanol, resuspended in isopropanol, and filtered. The final product was dried in a desiccator and the approximate yield was 75 %.

^1H NMR (D_2O , 500 MHz) δ 2.168 (2 H, m, CH_2), 2.93 (2 H, m, CH_2), 3.13 (3 H, s, CH_3), 3.51 (4 H, m, $(\text{CH}_2)_2$), 3.56 (2 H, m, CH_2), 3.97 (4 H, m, $(\text{CH}_2)_2$).

3.2.12 Cyclic-Sulfo betaines

3.2.12.1 CSB-1



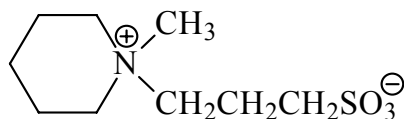
N-Methyl-N-(3-sulfopropyl)morpholinium, inner salt

CAS # 111282-24-7

1-Methylmorpholine (0.2 moles) and 1,3-propane sultone (0.2 moles), were mixed in 125 mL of dichloromethane. The mixture was covered and allowed to stand at room temperature. Crystals started appearing within 2 days. After 5 days, more dichloromethane was added and the solids were filtered out. These were resuspended in isopropanol and filtered. Finally the product was washed with isopropanol and n-propanol, filtered and sucked dry. The final approximate yield was 80 %.

^1H NMR (D_2O , 500 MHz) δ 2.18 (2 H, m, CH_2), 2.92 (2 H, t, J 8.3 Hz, CH_2), 3.15 (3 H, s, CH_3), 3.46 (4 H, m, $(\text{CH}_2)_2$), 3.55 (2 H, m, CH_2), 3.975 (4 H, s, $(\text{CH}_2)_2$).

3.2.12.2 CSB-2



N-Methyl-N-(3-sulfopropyl)piperidium, inner salt

CAS # 160788-56-7

1-Methylpiperidine (0.2 moles) and 1,3-propane sultone (0.2 moles), were mixed in 125 mL of dichloromethane. The mixture started depositing solids within 30 minutes of mixing. The mixture was covered and allowed to stand at room temperature for 4 days. More dichloromethane was added and the solids were filtered off. They were

resuspended in isopropanol and filtered. Finally the product was washed with isopropanol and n-propanol, filtered and sucked dry. The final product was slight pinkish in colour and the approximate yield was 85 %.

^1H NMR (D_2O , 500 MHz) δ 1.59 (2 H, m, CH_2), 1.81 (4 H, t, J 5.4 Hz, $(\text{CH}_2)_2$), 2.13 (2 H, m, CH_2), 2.90 (2 H, t, J 6.8 Hz, CH_2), 2.99 (3 H, s, CH_3), 3.29 (4 H, m, $(\text{CH}_2)_2$), 3.42 (2 H, m, CH_2).

Table 3.1: Results of elemental analysis of synthetic compensatory solutes, Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand.

Solute	Mol. Formula *	Mol.Wt.		Carbon %	Hydrogen %	Nitrogen %	Sulfur %
Propio betaine [PB]	$C_6H_{13}NO_2 \cdot H_2O$	149.19	Found	46.92	9.92	8.96	-----
			Calculated	48.30	10.13	9.39	-----
Deanol betaine [DB]	$C_6H_{13}NO_3$	147.17	Found	47.75	8.59	9.21	-----
			Calculated	48.97	8.90	9.52	-----
Homodeanol betaine [HDB]	$C_7H_{15}NO_3$	161.20	Found	50.04	8.81	8.25	-----
			Calculated	52.16	9.38	8.69	-----
Homoglycerol betaine [HGB]	$C_8H_{17}NO_4 \cdot H_2O$	209.24	Found	47.43	8.26	6.87	-----
			Calculated	45.92	9.15	6.69	-----
Diethanol homobetaine [DEHB]	$C_8H_{17}NO_4$	191.23	Found	48.72	8.68	7.04	-----
			Calculated	50.25	8.96	7.32	-----
Triethanol homobetaine [TEHB]	$C_9H_{19}NO_5 \cdot H_2O$	239.27	Found	45.64	8.56	6.66	-----
			Calculated	45.18	8.85	5.85	-----
CB-1	$C_8H_{15}NO_3 \cdot H_2O$	191.23	Found	48.37	8.40	6.86	-----
			Calculated	50.25	8.96	7.32	-----
CB-2	$C_9H_{17}NO_2 \cdot 2H_2O$	207.27	Found	52.61	9.17	6.68	-----
			Calculated	52.15	10.21	6.76	-----
CB-3	$C_8H_{15}NO_2 \cdot H_2O$	175.23	Found	52.73	9.51	7.61	-----
			Calculated	54.84	9.78	7.99	-----

Solute	Mol. Formula [*]	Mol.Wt.		Carbon %	Hydrogen %	Nitrogen %	Sulfur %
Dimethylthetin [DMT]	C ₄ H ₈ O ₂ S.H ₂ O	138.18	Found	34.89	7.29	-----	23.91
			Calculated	34.77	7.29	-----	23.20
SB-1	C ₆ H ₁₅ NO ₄ S	197.25	Found	35.41	7.77	6.88	16.24
			Calculated	36.54	7.66	7.10	16.25
SB-2	C ₇ H ₁₇ NO ₄ S	211.28	Found	39.82	8.04	6.69	15.09
			Calculated	39.79	8.11	6.63	15.27
SB-3	C ₈ H ₁₉ NO ₄ S	225.30	Found	42.23	8.30	6.19	13.84
			Calculated	42.65	8.50	6.22	14.23
SB-4	C ₈ H ₁₉ NO ₅ S	241.30	Found	39.58	7.81	5.78	13.49
			Calculated	39.82	7.94	5.80	13.29
CSB-1	C ₈ H ₁₇ NO ₄ S.H ₂ O	241.30	Found	41.56	8.02	6.25	13.81
			Calculated	39.82	7.94	5.80	13.29
CSB-2	C ₉ H ₁₉ NO ₃ S	221.31	Found	48.73	8.71	6.58	14.21
			Calculated	48.84	8.65	6.33	14.49

^{*}hydration of all solutes based on the results of elemental analysis

3.3 RESULTS AND DISCUSSION

The majority of the synthetic compensatory solutes were easy to synthesize and the yield was usually more than 75%. Applying the principles of a few existing methods, new solutes were synthesized. The general method of synthesis of these solutes is, the starting materials were mixed and the mixture left in the fume cupboard for a few days till the solids precipitated. To remove any starting materials or impurities in the product, all the solutes were washed several times with *n*-propanol, isopropanol and dichloromethane, and sucked dry, till a clear white crystalline powder was obtained. The structure and the composition of the products were confirmed by ^1H NMR and elemental analysis. Results of elemental analysis from Campbell Microanalytical Laboratory are as shown in Table 3.1. The laboratory claims that $\pm 0.3\%$ is the standard error of their results. Hydration state of each solute was estimated from the results of their elemental analysis.

Only two solutes, dimethylthetin and deanol betaine, were obtained as hydrohalides and rest of the solutes were obtained in a free base form. Solute obtained as a hydrohalide were converted to free base by applying to a Dowex 50 column (H^+ form) as described in section 3.2.1. The product was eluted from the column using 5.1 M ammonia solution. The final product was isolated from the eluant by evaporation under reduced pressure using a rotary evaporator.

Though the yield of most of the syntheses was good, low yields were observed for a few solutes such as DEHB, TEHB and HPHB. Different methods of synthesis were tried to increase the yield of the product. To prevent polymerization products being formed during syntheses of DEHB and TEHB, we tried adding hydroquinone in order to inhibit the polymerization reaction. Though adding hydroquinone resulted in better yields, the final product was brownish in colour and several attempts to remove this colour from the solutes were unsuccessful. It was later realised that these brownish solutes were not suitable for spectrophotometric methods of measuring enzyme activity, which involved following the change in absorbance at wavelengths in the near UV. Thus, hydroquinone was not added for further syntheses.

All the three cyclic betaines, CB-1, 2 & 3, were not difficult to synthesize and the products were obtained easily by just mixing the starting materials. However, impurities in the product being the main problem, all the three cyclic betaines had to be washed several times with solvents to remove the starting materials. Furthermore, to recover the final product, solids were suspended in isopropanol overnight and filtered. For unknown reasons, aqueous solutions of CB-2 and CB-3 were acidic and were destabilizing the enzymes during analytical measurements. As CB-2 and CB-3 were synthesized during the finishing stages of this project and due to the time constraints, attempts were not made to remove those acidic impurities. To prevent this problem in future syntheses, we suggest that the solutions of CB-2 and CB-3 should be passed through an anion exchanger and then recrystallized using a rotary evaporator, to get the final product.

SB-1 was synthesized using a method described by Barnhurst (1961) and the synthesis was complicated compared to other sulfobetaines. One of the starting materials, vinylsulfonic acid is available only in the form of 25% w/v vinylsulfonic acid sodium salt in water and to convert it into a free acid, concentrated HCl had to be added along with the starting materials. Concentrated HCl was again added during the later stages of the SB-1 synthesis to separate inorganic salts from the product. Due to these additions, the product contained a lot of acidic impurities and had to be resuspended in isopropanol several times to get the pure product. Even though SB-1 went through several cleaning steps, we still suspect that the final product contained a small percentage of impurities and this was evident by the inconsistent results obtained during different experiments using SB-1 (discussed in later chapters).

CSB-1 and CSB-2 were also synthesized during the later stages of the project, so were only used for DNA melting studies and not in enzyme stability experiments. Due to the difficulties faced during synthesis and very low yields, HPHB and TEHB have not been used in any of the experiments in this thesis. Also, CB-2 and CB-3, due to their strong acidic impurities, were not used in any of the experiments.

Table 3.2 lists the estimated costs of synthetic solutes described in this chapter. For comparison purposes, the cost estimation has been based on the raw materials required for the synthesis, using the prices of raw materials in the Sigma and Aldrich catalogues. The listed costs are an estimation based on a laboratory scale synthesis and do not take into account the cost of solvents required for the synthesis, which would be recovered in an industrial process. If an industrial process is designed for the syntheses of these solutes, the cost of the solutes would reduce as the raw materials would be purchased in bulk and syntheses would be in a large scale.

Table 3.2: Estimated cost of 500 g of synthetic compensatory solutes compared with price of 500 g of natural solutes based on the prices in the Sigma catalogue.

Synthetic Solutes

Natural Solutes – Sigma, MO, USA

Solute	Estimated cost of 500g (A \$)		Solute	Price of 500 g (A \$)
Propio betaine	66.40		Sorbitol	27.20
DB	678.40		Glycine betaine	164.00
HDB	45.90		Trehalose	959.00
HGB	1173.20			
DEHB	56.90			
TEHB	32.80			
HPHB	88.80			
DMT	88.10			
CB-1	385.20			
CB-2	123.40			
CB-3	165.70			
<u>Sulfobetaines</u>				
SB-1	189.10			
SB-2	1565.40			
SB-3	709.10			
SB-4	1381.70			
CSB-1	1642.10			
CSB-2	1575.10			

(Note: Costs of synthetic solutes are based only on the price of raw materials)

Comparing the estimated cost of synthetic solutes with the price of natural solutes (Table 3.2), we observe that a few solutes such as propio betaine, HDB, DEHB and DMT are inexpensive and are a fraction of the price of natural solutes. Solute DB and HGB are slightly expensive compared to other carboxylic acid solutes.

The sulfonic acid analogues or sulfobetaines are expensive as well due to the high cost of their raw materials. However, the costs listed in Table 3.2 are based only on a laboratory scale synthesis, and designing a proper large scale process for the syntheses of these expensive solutes could reduce their cost substantially.

3.4 CONCLUSIONS

We conclude that synthetic compensatory solutes described in this chapter are easy to synthesize and a few are inexpensive compared to the natural solutes available in the market. Though impurities in the final products pose a problem, design and optimization of a proper synthesis method could result in a better and pure product. These novel solutes have paved a way for synthesis of more inexpensive synthetic solutes for specific applications.

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Chapter 4 Protein Stabilization Using Synthetic Compensatory Solutes

Part A

4.1 Stabilization of Catalytic Activity of Different Model Enzymes

4.1.1 Introduction

The loss of catalytic activity of enzymes limits their use in biotechnological processes. Most enzymes are easily unfolded by changes in conditions such as heat, changes in pH or in the presence of chaotropic agents. As discussed in previous chapters, in nature, many living cells protect their enzymes by accumulating compounds such as methylamines, amino acids, polyols and sugars. *In vitro*, laboratory workers have been trying to stabilize enzymes against high and low temperatures by adding those compounds to enzyme solutions at different concentrations. However, the stabilizing effect of these compounds can sometimes be enzyme or stress specific, and their effectiveness cannot be generalized. There is a need for more compounds or a range of stabilizing compounds which are inexpensive and have better stabilizing properties than most of the known natural solutes.

In a study of thermal destabilization of RNase A by urea, glycine betaine and glycerophosphocholine were found to be very good stabilizers of the enzyme (Burg and Peters, 1998). Kaushik and Bhat (1998) studied the effects of a series of carboxylic acid salts on the thermal stability of five different proteins that vary in their physico-chemical properties. They found that there was a linear correlation between the increase in thermal stability and the number of carboxylic groups in the salt. Sugars have been shown to protect proteins against thermal denaturation. Among several sugars, trehalose, has been known to be a superior stabilizer in providing protection to biological materials and has been found to be very effective for stabilization of proteins on exposure to high temperatures in solution (Lin and Timasheff, 1996; Xie and Timasheff, 1997). Trehalose

and glycerol have been used to stabilize and renature yeast inorganic pyrophosphatase inactivated by very high temperatures (Zancan and Sola-Penna, 2005).

Lipper and Galinski (1992) studied the effects of compatible solutes, including two new natural solutes, ectoine and hydroxyectoine, on phosphofructokinase (PFK) and lactate dehydrogenase (LDH) against different stresses. During thermal destabilization of the enzymes, they showed that hydroxyectoine, trehalose and betaine were able to increase the T_{D-50} , temperature at which 50% of enzyme activity is lost, by almost 14°C. Differential scanning calorimetry (DSC) study on thermal unfolding of bovine RNase A demonstrated that hydroxyectoine increased the melting temperature of RNaseA significantly and proved to be a very good protein stabilizer (Knapp et al., 1999).

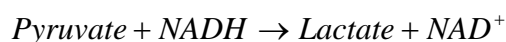
Protection of enzymes against low temperatures, freeze-thawing and freeze-drying, has also been an important area of research for biotechnologists. During freeze-thawing and freeze-drying, proteins have to endure stresses such as low temperatures and formation of ice, which destabilize them. A variety of solutes, including sugars, polyols, amino acids and methylamines have been examined for their effects on retention of enzyme activity upon freeze-thawing and freeze-drying (Carpenter and Crowe, 1989). During freeze-thawing, several compounds that stabilize proteins in their solution state protect them against low temperatures, but during freeze-drying sugars have been found to protect proteins (Carpenter et al., 1987; Carpenter and Crowe, 1989). Hydroxyectoine, with a free OH group, has also been shown to be an effective stabilizer against freeze-drying (Lippert and Galinski, 1992).

Of the variety of enzyme stabilizers known, trehalose, a disaccharide, appears to be an effective protectant against both high and low temperatures, both *in vivo* and *in vitro*. A number of hypotheses have been proposed to explain the stabilizing property of trehalose. Carpenter and Crowe (1989) proposed that sugars like trehalose act as water substitutes by replacing the hydrogen bonds during freeze-drying, thus protecting the proteins. Work by Kaushik and Bhat (2003) has shown a decrease in the heat capacity of protein denaturation (ΔC_p) in trehalose solutions, and that there was a direct correlation of the

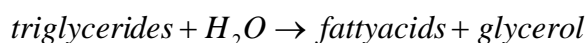
surface tension of trehalose solutions and the thermal stability of various proteins. They concluded that trehalose can be expected to work as a universal stabilizer of proteins. In a more recent study of trehalose-protein interaction in aqueous solution (Lins et al., 2004), it was proposed that trehalose molecules cluster and move toward the protein, but neither completely expel water from the protein surface nor form hydrogen bonds with the protein.

Even though trehalose is an exceptional and possibly a universal stabilizer, it is expensive to purchase and this limits its application. It has always been of interest to find inexpensive alternatives to expensive stabilizers. In this chapter we explore the stabilization effect of our inexpensive synthetic compensatory solutes on three different model enzymes against different stress factors. The enzymes considered are, lactate dehydrogenase (LDH) (from rabbit muscle), α -chymotrypsin (from bovine pancreas) and a microbial lipase. These enzymes were subjected to different stresses such as heat denaturation, freeze-thawing and freeze-drying, and the protective effect of novel synthetic solutes was assessed.

Lactate dehydrogenase (LDH) is a tetrameric enzyme belonging to the class of oxidoreductases and catalyzes the conversion of pyruvate to lactate. LDH can be easily denatured and has been previously used to study the effect of natural compensatory solutes on enzyme stability (Goller and Galinski, 1999). The reaction catalyzed by LDH can be represented as:



α -Chymotrypsin is a protease which catalyses the hydrolysis of peptide bonds and ester bonds. It is a trimeric enzyme usually active in the digestive system of mammals and other organisms. Lipase is an enzyme which catalyzes the hydrolysis or formation of lipids. Lipases are being increasingly employed for biotechnological processing and are used in many industrial applications (e.g. detergents, oils and fats, leather and paper). The reaction catalyzed by lipase can be represented as:



4.1.2 Materials and Methods

4.1.2.1 Materials

L-Lactate dehydrogenase (LDH) (type XI: from rabbit muscle, EC 1.1.1.27), α -chymotrypsin (type II: from bovine pancreas, EC 3.4.21.1), pyruvate, *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide, 1,4 – dioxane, NADH, trehalose, sorbitol and glycine betaine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lipase (type VII: from *Candida rugosa*, EC 3.1.1.3) was purchased from Sigma, Japan. KH_2PO_4 , K_2HPO_4 , Na_2HPO_4 and phosphate buffer tablets (pH 7.2) were obtained from BDH (Poole, England). Tributyrin (98%) was purchased from Aldrich Chemical Co., USA.

4.1.2.2 Equipment

LDH activity and chymotrypsin activity was measured on a UV spectrophotometer (Cary 4000, Varian, Melbourne, Australia) equipped with a temperature controlled cuvette holder. Lipase activity was measured using an autotitrator (DL50, Mettler Toledo) with a sensor (DG113, Mettler Toledo). Freeze drying was carried out in a general purpose freeze dryer (Model 24DX24, Virtis).

4.1.2.3 LDH activity assay

LDH activity was measured at 32°C. All solutions were made in 50 mM potassium phosphate buffer, pH 7.5. The 2.0 ml reaction mixture contained 2.3 mM pyruvate and 0.26 mM NADH. The spectrophotometer was blanked with the reaction mixture and the reaction was initiated by the addition of 50 μL of LDH solution (10 $\mu\text{g}/\text{mL}$) and was followed by measuring the decrease in absorbance at 340 nm. The reaction mixture was held in a temperature controlled cuvette holder for five minutes prior to the addition of enzyme.

4.1.2.4 Chymotrypsin activity assay

Chymotrypsin activity was measured at room temperature (22°C). The substrate, *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide, was prepared in 1,4-dioxane solution. The 1 ml reaction mixture contained 50 mM sodium phosphate buffer, pH 8.0, 0.4 mg/mL of enzyme and 0.15 mM substrate. The spectrophotometer was blanked before the addition of substrate. The reaction was initiated by the addition of substrate and was monitored by observing the increase in absorbance at 405 nm.

4.1.2.5 Lipase activity assay

Lipase activity was measured at room temperature (22°C). The reaction mixture contained 1 mL tributyrin in 25 mL phosphate buffer (5 mM, pH 7.2, prepared from buffer tablets, BDH, Poole, England). A pretitration run was done to attain the set pH before the final titration. The titrant used was 0.05 N NaOH. The reaction was initiated by adding 100 µL of lipase solution (0.3% w/v) and the reaction was run for 10 min. The lipase activity was calculated from the amount of NaOH consumed (ie the fatty acids produced).

Stress experiments

After determining the catalytic activity of the enzyme, aliquots of the enzyme solution were transferred to microcentrifuge tubes, supplemented with compensatory solutes (final concentration: 1M) and then subjected to heat denaturation or freeze drying. The total volume of enzyme/stabilizer solution was 250 µL for LDH and chymotrypsin assay, and 1000 µL for lipase assay.

In controls, the enzymes were incubated with compensatory solutions (final concentration: 1M) for 10 min at room temperature (22°C). The activities of the enzymes were then determined as described above. The relative activity of the enzymes was expressed as a percentage of the activity prior to the stress experiments.

4.1.2.6 Heat denaturation

For heat denaturation, the enzyme/stabilizer solutions in microcentrifuge tubes were incubated in a temperature block for 10 min at the specified temperature. The holes of the temperature block were partially filled with water to ensure good conductivity of temperature to the tubes.

4.1.2.7 Freeze-drying

For freeze drying, the enzyme/stabilizer solutions were frozen at approximately -50°C for two minutes using dry-ice in acetone followed by lyophilization for 24 hours at a temperature of -20°C using the freeze dryer. The enzymes were rehydrated using the same volume of distilled water as the mixture was before freeze-drying.

4.1.2.8 Freeze-thawing

For freeze-thawing, the enzyme/stabilizer solutions were frozen at approximately -50°C for two minutes using dry-ice in acetone and then thawed in a water bath at a constant temperature of 32°C. The same procedure was repeated for each freeze-thaw cycle.

4.1.2.9 Data analysis

The non-linear regression function of SigmaStat for Windows (v3, SPSS) was used to fit the relationship between the enzyme activity (% initial) and temperature to several empirical equations, using the Marquart-Levenberg algorithm. These equations were mainly used for interpolation to get T_{D-50} values, the temperature at which the enzyme loses 50% of its activity.

Empirical equations that gave excellent fits were a double exponential equation:

$$y = 100 * 2^{-e^{m(x-t)}} \quad (4.1)$$

a logistic equation:

$$y = \frac{100}{(1 + (\frac{x}{t})^m)} \quad (4.2)$$

and a sigmoid equation:

$$y = \frac{100}{(1 + e^{m(x-t)})} \quad (4.3)$$

where 'y' is the relative activity of the enzyme (% initial), 't' is the temperature (°C) at which the enzyme lost 50% activity and 'x' is the temperature. The parameter 'm' is a measure of the steepness of the curve. All data were fitted to all three equations. Equation 4.1 gave the best fit for heat denaturation of LDH and chymotrypsin, and equation 4.2 for heat denaturation of lipase.

Replicate trials (three) were conducted to observe the decrease in the activity of the enzymes with the increase in temperature and in the presence of different compensatory solutes. Parameters for each set of data were estimated by curve fitting, carried out with weights $1/(1+s^2)$ where s is the standard error of the mean of the activities at each temperature.

4.1.3 Results

4.1.3.1 Control experiments

The activity of the enzymes was not affected by pre-incubation with 1M solute solutions at room temperature. With all the solutes tested (Table 4.1), activity after incubation was >96% of controls without solute, for lactate dehydrogenase, chymotrypsin and lipase. Based on triplicate experiments, these results were not significantly different from 100%.

4.1.3.2 Heat Protection

All the compensatory solutes considered were able to protect the enzymes against heat when compared to the absence of compensatory solutes. The data were fitted to a few empirical equations (section 4.1.2.9) to estimate the values of T_D-50 for each enzyme in

the presence and absence of compensatory solutes during heat denaturation. The double exponential equation gave the best fit for LDH and chymotrypsin, and logistic equation gave the best fit for lipase. The results of estimates of T_{D-50} are shown in Table 4.1.

In case of LDH, though all the compensatory solutes were able to protect the enzyme against heat, synthetic solutes HDB and HGB gave the best protection with the T_{D-50} value of LDH being very high in their presence. In the absence of any solute solution, 50% of LDH activity is lost at $50.9 \pm 0.4^{\circ}\text{C}$, whereas in the presence of 1M HGB it increased to $59.5 \pm 1.0^{\circ}\text{C}$, an approximate increase of 9°C . Similar heat protection of LDH were observed in the presence of propio betaine, HDB, DEHB and CB-1, which increased the T_{D-50} by more than 7°C .

Of the three natural compensatory solutes considered, trehalose gave the best protection to LDH by increasing the T_{D-50} by almost 5°C . Sorbitol and GB gave a moderate heat protection to LDH with an increase in T_{D-50} by just 2.1°C and 4.4°C respectively. All the sulfobetaines considered were able to protect LDH against heat, but their protection was not similar to that of HGB or HDB. Of the sulfobetaines, SB-3 was the best stabilizer with an approximate 4.6°C increase in T_{D-50} (Table 4.1).

During heat denaturation of chymotrypsin, in the absence of any compensatory solute the enzyme lost 50% of its activity at $41.5 \pm 0.5^{\circ}\text{C}$ (Table 4.1). The best heat protection was observed in the presence of 1M HGB which increased the T_{D-50} of chymotrypsin to $50.9 \pm 0.6^{\circ}\text{C}$, an approximate increase of 9.5°C . Trehalose with its 5.2°C increase in T_{D-50} gave the best protection of all the three natural solutes. None of the other synthetic solutes were able to protect chymotrypsin against heat as effectively as HGB. Though HDB increased the T_{D-50} by almost 6°C , it was still 3.5°C less than HGB. There was no apparent change in the T_{D-50} of chymotrypsin in the presence of DEHB. Of the sulfobetaines considered, SB-3 was again the best stabilizer with a 5.5°C increase in T_{D-50} . SB-1 did not seem to protect chymotrypsin against heat as it increased the T_{D-50} by just 1°C .

Table 4.1: Estimates of $T_D-50 \pm \text{SEM}$ (from three trials), temperature at which enzymes lost 50% activity in the presence of different compensatory solutes (1M). Approx. changes in T_D-50 are obtained by comparing individual T_D-50 values with that of no addition.

	Lactate dehydrogenase			Chymotrypsin			Lipase	
Solute	T_D-50, °C	Change in T_D-50, °C		T_D-50, °C	Change in T_D-50, °C		T_D-50, °C	Change in T_D-50, °C
No addition	50.9 ± 0.4	0.0		41.5 ± 0.5	0.0		54.9 ± 0.9	0.0
Trehalose	55.8 ± 0.0	+ 4.9		46.7 ± 0.5	+ 5.2		67.5 ± 0.4	+ 12.6
Sorbitol	53.0 ± 0.6	+ 2.1		45.1 ± 0.2	+ 3.6		58.9 ± 0.8	+ 4.0
Glycine betaine	55.3 ± 0.2	+ 4.4		45.9 ± 0.3	+ 4.4		55.2 ± 0.8	+ 0.3
Propio betaine	58.7 ± 0.4	+ 7.8		44.5 ± 0.2	+ 3.0		-----	-----
DMT	54.3 ± 0.1	+ 3.4		45.4 ± 0.6	+ 3.9		55.5 ± 0.8	+ 0.6
CB-1	58.7 ± 0.3	+ 7.8		45.0 ± 0.6	+ 3.5		-----	-----
DB	54.6 ± 0.3	+ 3.7		46.0 ± 0.5	+ 4.5		-----	-----
HDB	59.2 ± 0.5	+ 8.3		47.4 ± 0.4	+ 5.9		66.4 ± 0.4	+ 11.5
HGB	59.5 ± 1.0	+ 8.6		50.9 ± 0.6	+ 9.4		-----	-----
DEHB	57.9 ± 0.4	+ 7.0		41.3 ± 0.8	- 0.2		-----	-----
SB-1	54.3 ± 0.9	+ 3.4		42.6 ± 0.8	+ 1.1		-----	-----
SB-2	55.2 ± 0.6	+ 4.3		46.9 ± 0.3	+ 5.4		-----	-----
SB-3	56.2 ± 0.4	+ 4.6		47.0 ± 0.7	+ 5.5		-----	-----
SB-4	54.2 ± 0.5	+ 3.3		46.0 ± 0.2	+ 4.5		-----	-----

Heat Denaturation of LDH

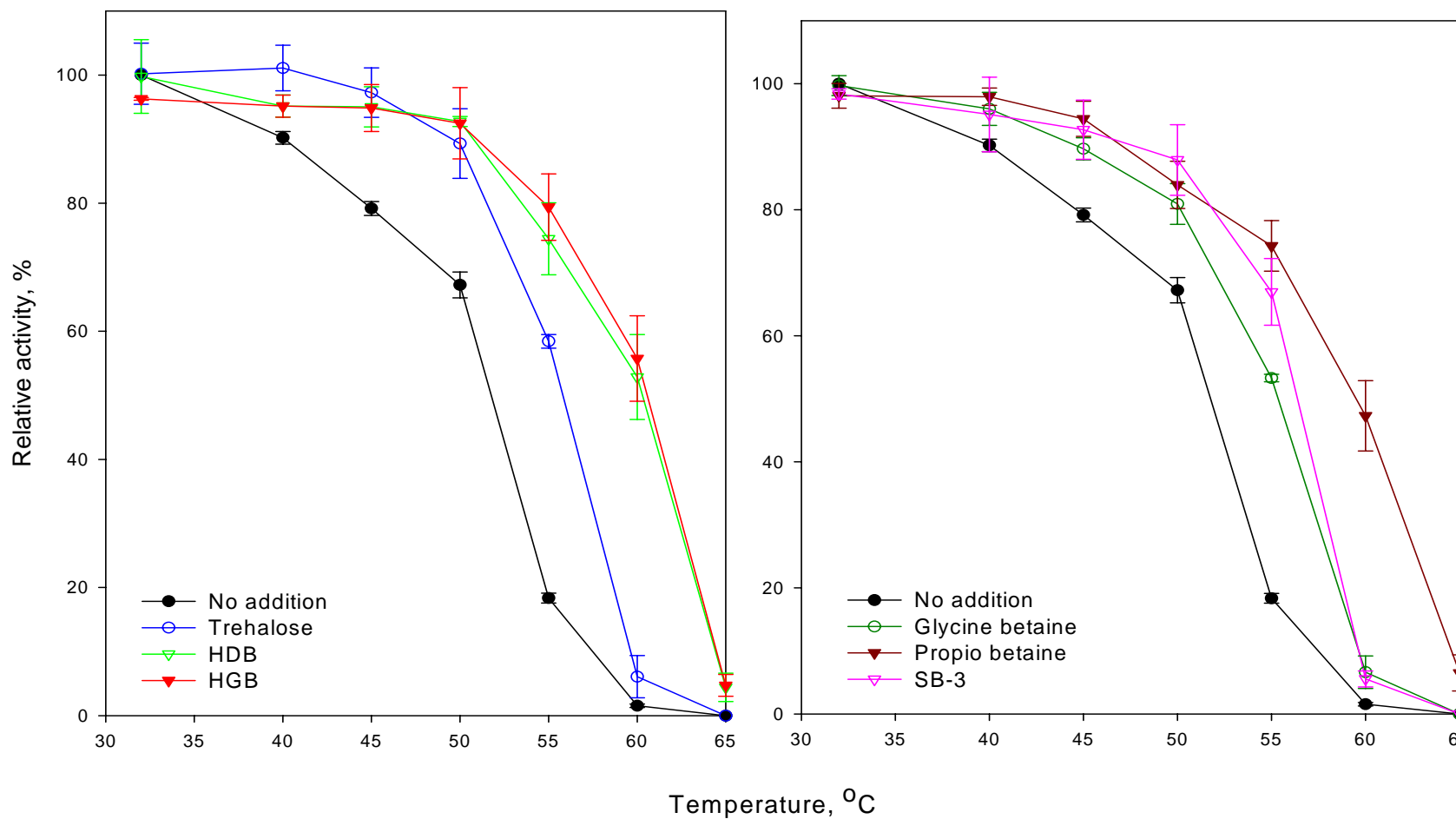


Figure 4.1: Effect of heat (10-minute pre-incubation at each temperature) on activity of lactate dehydrogenase (LDH) in the presence of natural and synthetic compensatory solutes.

Heat Denaturation of Chymotrypsin

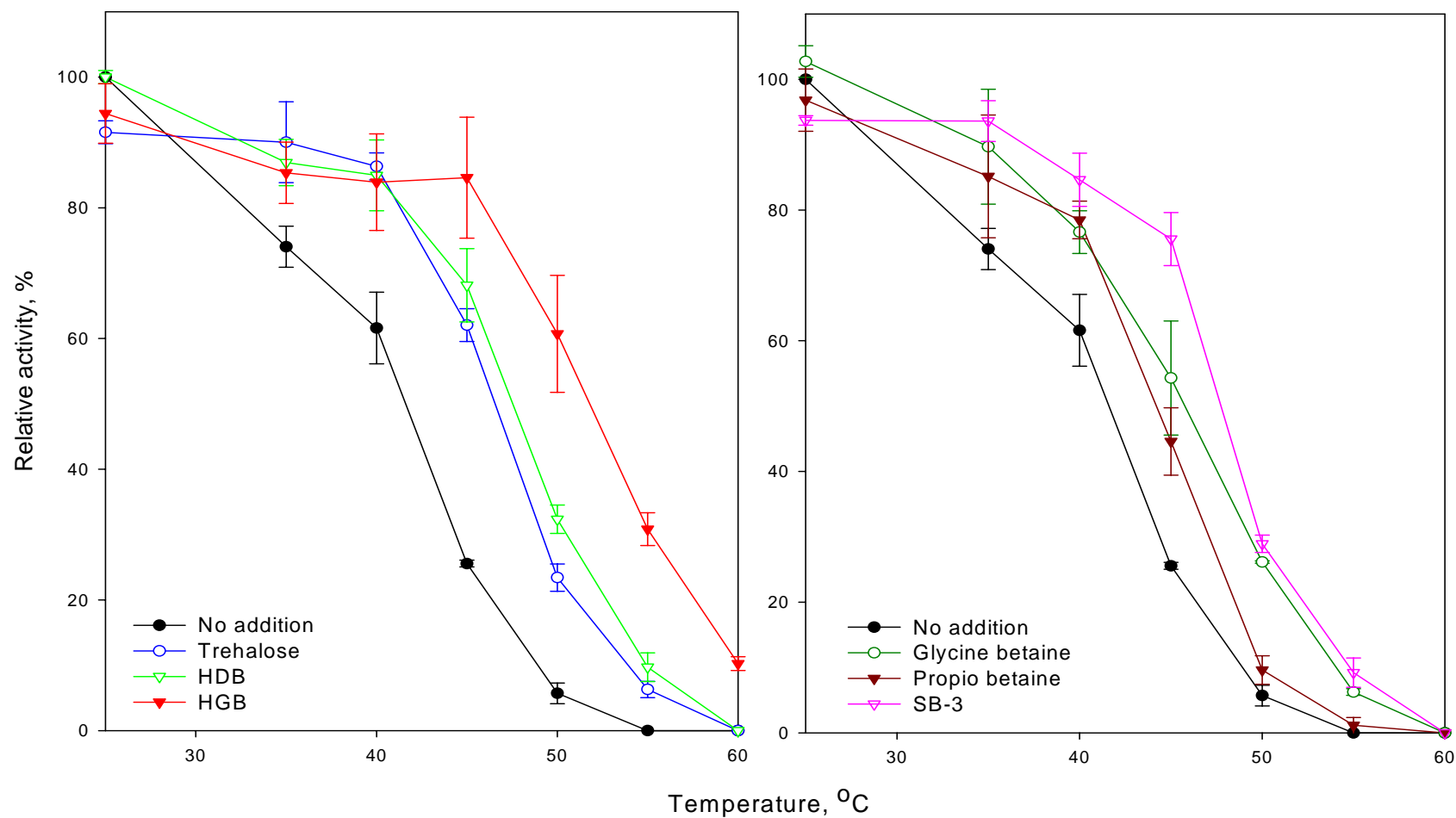


Figure 4.2: Effect of heat (10-minute pre-incubation at each temperature) on activity of α -chymotrypsin in the presence of natural and synthetic compensatory solutes.

Experiments on heat denaturation of lipase were conducted during the early stages of the project and only two synthetic compensatory solutes, HDB and DMT, were considered for the study. With the application of heat, lipase lost 50% of its activity at $54.9 \pm 0.9^\circ\text{C}$ in the absence of compensatory solutions. Increases in T_{D-50} were not significant with glycine betaine and dimethylthetin, though sorbitol and especially trehalose and HDB significantly enhanced stability (Table 4.1 and Figure 4.3). The maximum increases were between 9 and 13 degrees, and were in the presence of either the natural stabilizer trehalose or with synthetic solute HDB.

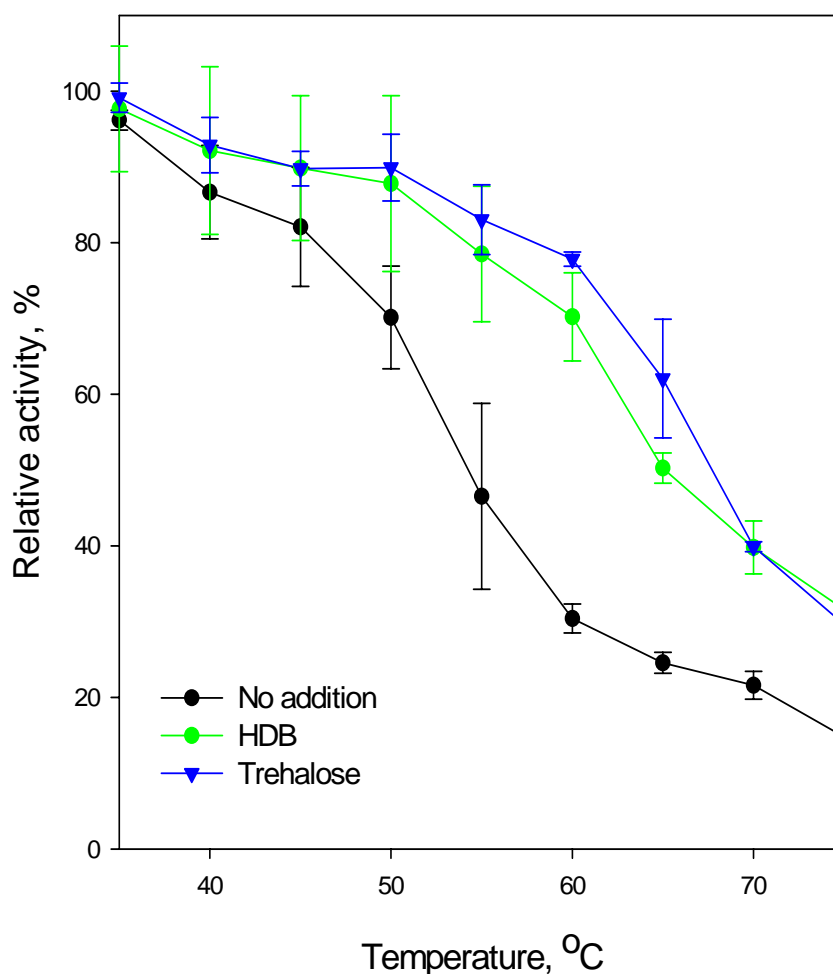


Figure 4.3: Heat Denaturation of Lipase: effect of heat (10-minute pre-incubation at each temperature) on activity of lipase in the presence of HDB and trehalose.

The rates at which the activity of the enzymes is lost in the presence of different compensatory solutions are shown in Figure 4.1, 4.2 and 4.3. During heat denaturation of LDH (Figure 4.1) at 60°C, the relative activity (%) in the presence of most of the compensatory solutes is less than 10%. However, at 60°C, in the presence of synthetic solutes HDB and HGB, the relative activity is more than 50%, and between 25% and 50 % in the presence of DEHB, CB-1 and propio betaine. Even in the presence of the most effective natural stabilizer, trehalose, only 6% of the initial activity LDH is retained at 60°C. The results show that in the presence of synthetic stabilizers such as HDB and HGB, LDH activity was lost slowly with the increase in temperature and at extreme temperatures more activity was retained in their presence than in the presence of any of the other solutes.

Similarly, during heat denaturation of chymotrypsin (Figure 4.2), HGB shows better protection at higher temperatures compared to other solutes. At 55°C, relative activity in the presence of HGB is more than 30%, whereas it is less than 10% in the presence of most of the other solutes. The same trend is observed in case of lipase (Figure 4.3), where synthetic solute HDB seems to be a better stabilizer than trehalose at extreme temperatures.

4.1.3.3 Protection against Freeze-drying

Two enzymes, LDH and lipase, were used to study the protective effects of compensatory solutes against freeze-drying. In the case of LDH, the enzyme could retain 26% of its activity in the absence of any compensatory solute (Figure 4.4). Trehalose was able to effectively stabilize LDH by helping it retain almost 80% of its initial activity. In the presence of synthetic solutes HGB and HDB, LDH retained around 63% and 42% of its initial activity respectively. DB, DEHB, SB-2 and sorbitol showed some moderate stabilizing effect, and were able help LDH retain more than 30% of its activity. However, on a molar basis none of the synthetic solutes were as effective as trehalose. Propio betaine, GB, DMT, SB-1 and SB-3 did not confer any protection to LDH during freeze-drying.

Freeze-drying of LDH

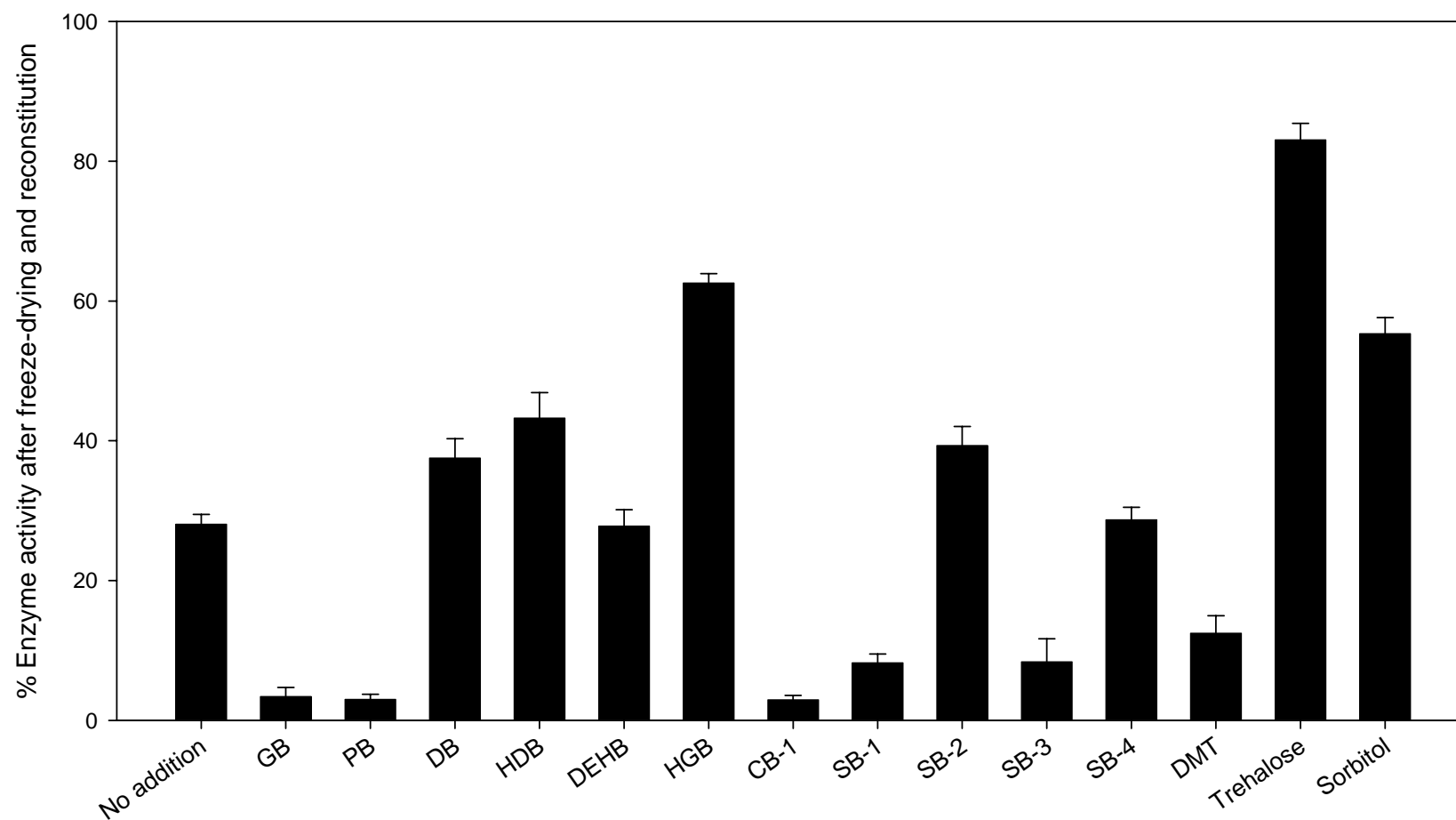


Figure 4.4: Relative activity (%) of LDH after freeze-drying and reconstituting enzyme solutions containing 1 M different solutes.

Of the sulfobetaines, SB-2 gave the best protection to LDH by helping it retain almost 40% of its initial activity. Surprisingly, SB-1 and SB-3 did not protect LDH during freeze-drying, and seemed to destabilize the enzyme as the relative activity was less than the no addition. Most of the synthetic solutes containing OH groups in their structure were able to stabilize LDH against freeze-drying, except SB-1 and SB-3. Relative activity in the presence of solutes without hydroxyl group such as propio betaine, GB, DMT and CB-1, was less than the relative activity found in the absence of any solutes (no addition).

The best synthetic solutes to protect LDH during freeze-drying, HDB and HGB, were mixed with trehalose in various molar proportions and their effect on LDH during freeze-drying was assessed. Results are as shown in Figure 4.5.

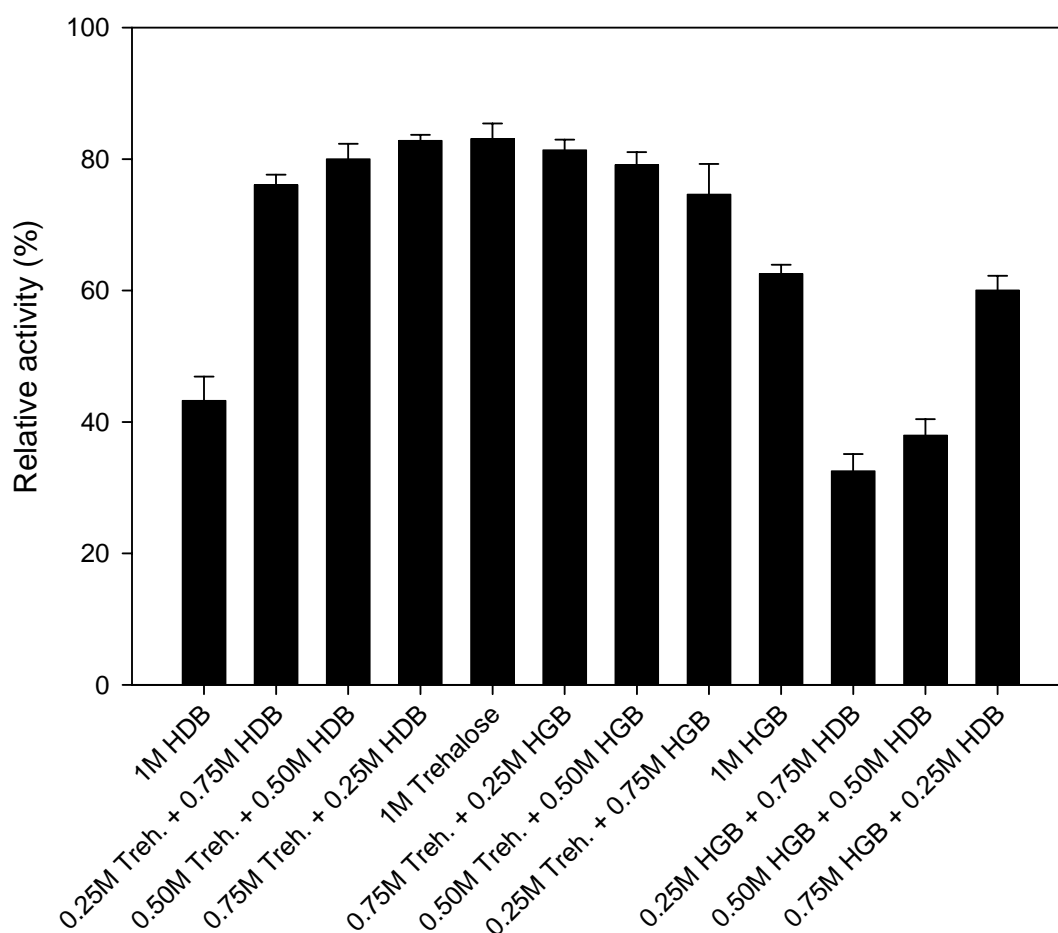


Figure 4.5: Freeze-drying of LDH with mixtures of compensatory solutes.

No significant results were obtained when the solutes were mixed during freeze-drying. The effect of trehalose seemed to be more dominant in all the mixtures. One interesting results was that mixtures containing 0.75 M HDB + 0.25 M trehalose and 0.75 M HGB + 0.25 M trehalose gave a similar stabilization effect as 1 M trehalose.

As mentioned earlier, lipase experiments were conducted during the early stages of the project and only two synthetic solutes, HDB and DMT, were used. Lipase, an enzyme known for its stability at low temperatures, was able to retain almost 60 % of its activity even in the absence of stabilizers. Trehalose and HDB significantly increased the stability, enabling lipase to retain more than 80% activity (Figure 4.6). Glycine betaine, sorbitol and DMT did not significantly affect the stability of lipase during freeze drying.

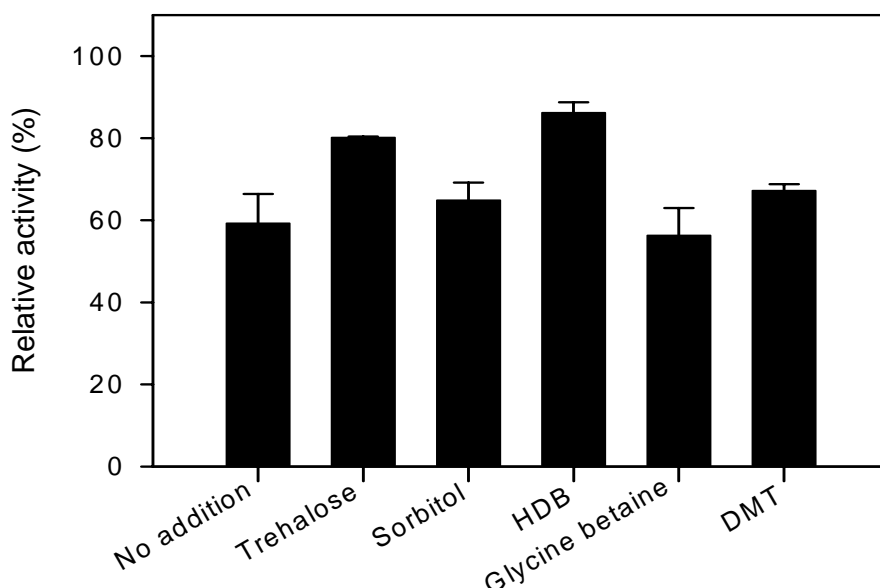


Figure 4.6: Relative activity (%) of lipase after freeze-drying and reconstitution.

Trehalose significantly stabilized both LDH and lipase against freeze drying. Compared to other synthetic solutes HGB was a very effective stabilizer of LDH during freeze-drying and showed better stabilizing effect than sorbitol (a polyol). In the case of lipase, synthetic solute HDB was as effective as trehalose, but not as effective for stabilizing LDH.

Furthermore, HDB and HGB have approximately half the molecular weight of trehalose and thus the mass of these synthetic solutes used in the experiments was less.

4.1.3.4 Protection against Freeze-thawing

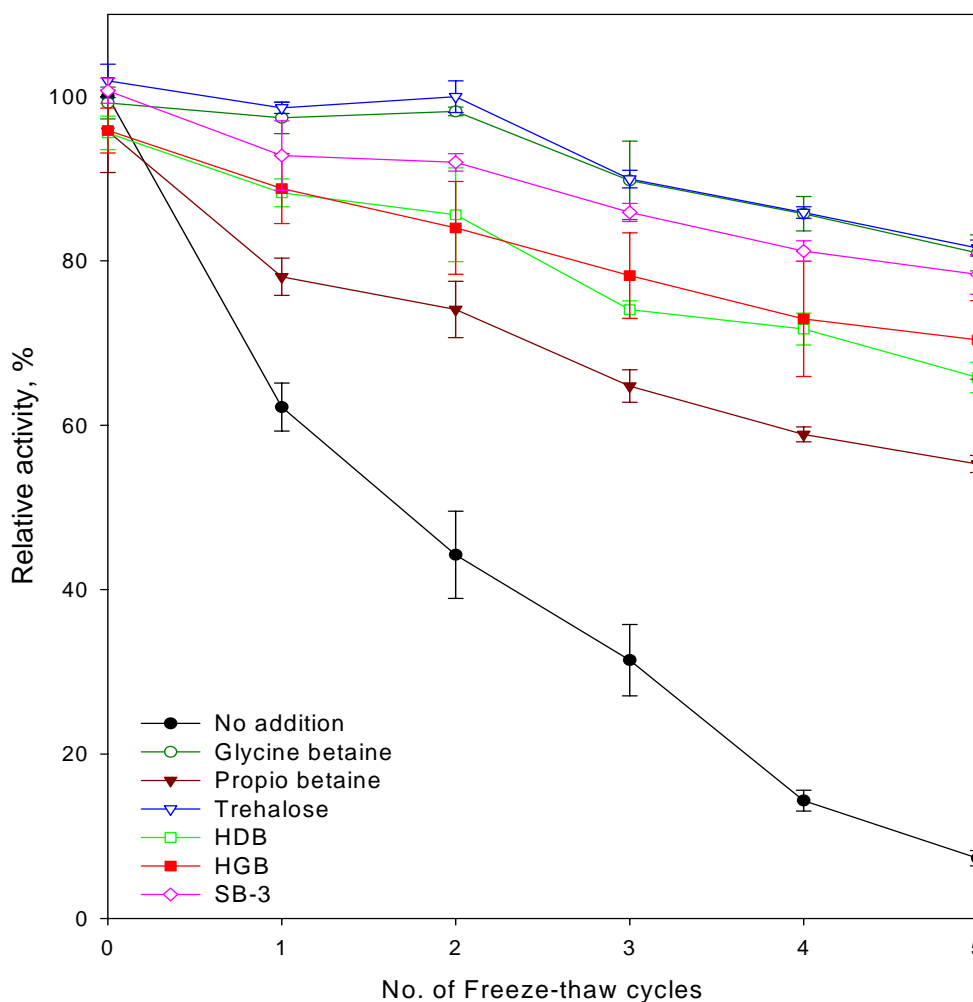


Figure 4.7: Relative activity (%) of lactate dehydrogenase after different number of freeze-thaw cycles in the presence of 1M compensatory solutes.

Only LDH was used to study the effect of compensatory solutes during freeze-thawing. All the compensatory solutes considered, both natural and synthetic, were able to confer protection to LDH during freeze-thawing. In the absence of stabilizers, LDH lost 50% of its activity after two freeze-thaw cycles and by the end of fifth cycle, only 7 % of the initial

activity was retained (Table 4.2). Natural solutes, GB, sorbitol and trehalose, gave better stabilization compared to synthetic solutes. Even after five cycles, more than 80 % of the initial activity was maintained in the presence of natural solutes. Relative activity was between 50% and 80% in the presence of synthetic solutes after five freeze-thaw cycles. SB-1 was the only sulfobetaines that was unable to protect LDH against freeze-thawing. In the presence of other three sulfobetaines, SB-2, SB-3 and SB-4, more than 70% of the initial activity was retained after five cycles.

Table 4.2: Relative activity of LDH, after 1st and 5th freeze-thaw cycle, in the presence of 1 M compensatory solutes. All values quoted \pm SEM are from three replicate trials.

Solute	Relative activity (%) after no. of freeze-thaw cycles	
	1st cycle	5th cycle
No addition	62.2 \pm 2.9	7.3 \pm 0.9
Trehalose	98.6 \pm 0.7	81.6 \pm 1.0
Sorbitol	96.5 \pm 2.0	85.5 \pm 2.4
Glycine betaine	97.4 \pm 1.9	80.9 \pm 2.2
Propio betaine	78.1 \pm 2.2	55.3 \pm 1.0
DMT	90.3 \pm 2.1	66.3 \pm 2.0
CB-1	82.6 \pm 2.4	51.8 \pm 3.5
DB	92.6 \pm 1.5	74.6 \pm 2.0
HDB	88.3 \pm 1.7	65.8 \pm 1.8
HGB	88.8 \pm 4.2	70.4 \pm 4.8
DEHB	78.6 \pm 5.2	57.4 \pm 1.9
SB-1	76.6 \pm 4.0	51.8 \pm 3.0
SB-2	84.2 \pm 2.2	72.6 \pm 1.4
SB-3	92.8 \pm 4.2	78.3 \pm 2.4
SB-4	86.3 \pm 6.8	70.6 \pm 7.8

4.1.4 Discussion

Natural compensatory solutes have been exploited to stabilize enzyme activities, both zwitterionic solutes (Lippert and Galinski, 1992; Galinski, 1993; Goller and Galinski, 1999; Burg and Peters, 1998) and polyhydric solutes (Carpenter and Crowe, 1988a&b) have been used for this purpose. Carboxylic acid salt solutions (Kaushik and Bhat, 1998) and trehalose (Kaushik and Bhat, 2003) have been shown to increase the thermal stability of proteins, and trehalose has been judged as an exceptional and a universal protein stabilizer (Kaushik and Bhat, 2003). In this first part of the chapter we have tried to assess the protective effects of novel synthetic compensatory solutes on three different enzymes against high and low temperatures. Our results show that some of the synthetic compensatory solutes have better stabilizing properties than most the well known natural stabilizers. We chose heat denaturation, freeze-thawing and freeze-drying as the stress factors, and LDH, α -chymotrypsin and lipase as our model enzymes. As α -chymotrypsin is stable at low temperatures it was not used for freeze-thawing and freeze-drying experiments. Only two synthetic solutes, HDB and DMT, were used for lipase experiments, as the experiments were conducted during the early stages of this project.

Synthetic solutes, HGB and HDB, showed superior performance as thermoprotectants for both LDH and chymotrypsin, and effect of HDB was in par with trehalose for thermostabilization of lipase. Compared to trehalose, synthetic solutes were better performers as they helped the enzymes to retain their catalytic activity even at extreme temperatures. However, the degree of thermostabilization by synthetic solutes varied between the two enzymes. The temperature at which enzymes lost 50 % of their activity, T_{D-50} values, helped us to compare the stabilization effect of different solutes on different enzymes.

HDB and HGB were very good stabilizers during thermostabilization of LDH and had similar protective effect on the enzyme. However, in case of chymotrypsin the effect of HDB was comparatively lesser than HGB. A similar pattern was observed with propiono

betaine and CB-1, as both were better stabilizers while protecting LDH against heat, but had a very less effect on chymotrypsin. Interestingly, DEHB, which was a very good stabilizer of LDH against heat (increasing the T_{D-50} by almost 7°C) had absolutely no effect on chymotrypsin. HGB was the only solute which gave a consistent thermoprotection to both LDH and chymotrypsin during heat denaturation.

Natural stabilizers, GB, sorbitol and trehalose, were consistent in protecting the enzymes against heat. Trehalose showed considerably better stabilizing effect on lipase compared to GB and sorbitol. The T_{D-50} values of LDH in the presence of trehalose and GB are comparable to the results obtained by Lippert and Galinski (1992). In the same study, they examined the protection of LDH against heat stress by two novel compatible solutes, ectoine and hydroxyectoine, and showed that the protecting effect was pronounced in the case of hydroxyectoine, a solute containing hydroxyl group in its structure. Comparing those results with ours, it is observed that HDB and HGB seem to be better stabilizers than ectoines. To confirm these results, a single trial experiment of heat denaturation of LDH in the presence 1 M ectoine and hydroxyectoine (gifted by Prof. Erwin Galinski) was carried out. Results of these experiments showed that though ectoine and hydroxyectoine were able to protect LDH against heat (Figure 4.8), their effect was less significant compared to synthetic solutes HDB and HGB.

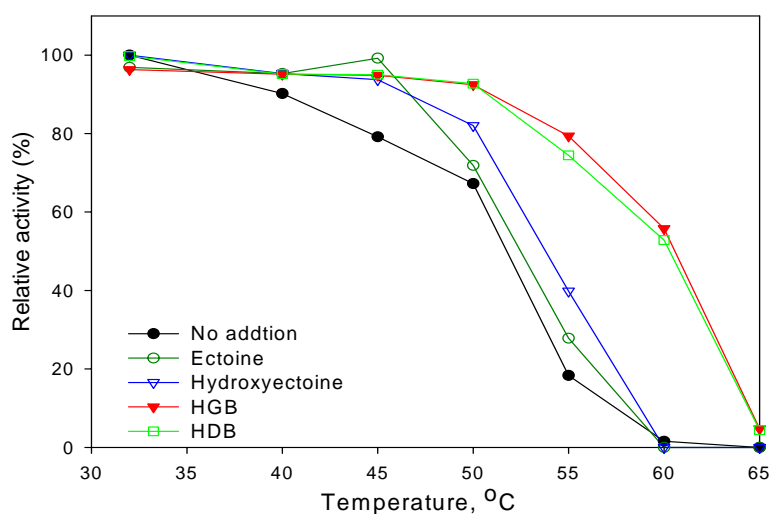


Figure 4.8: Effect of ectoine, hydroxyectoine, HDB and HGB against heat denaturation of LDH.

Except for SB-1, sulfobetaines were consistently able to stabilize both the enzymes against heat. SB-1 showed some thermoprotection to LDH, but had much less effect on chymotrypsin, which may be due to the impurities present in SB-1. Of the four sulfobetaines, SB-2 and SB-3 were good thermoprotectants of the enzymes and their effect was similar to that of trehalose. SB-3 seemed to be a slightly better thermostabilizer than SB-2.

Glycine betaine is known as a simple and useful cryoprotectant that works for cryopreservation of a wide range of prokaryotic organisms (Cleland et al., 2004). Glycine betaine and a few simple modified betaines have been shown to be effective cryoprotective additives (Lloyd et al., 1992; Lloyd et al., 1994). Trehalose has also been shown to be a good cryoprotectant during freeze-thawing of LDH (Galinski, 1993). It was found that all compensatory solutes considered in our study, synthetic and natural, gave significant protection to LDH during freeze-thawing (Table 4.2 and Figure 4.7). Compensatory solutes helped LDH retain more than 50% of its initial activity even after five freeze-thaw cycles. Our results show that natural solutes are better stabilizers than synthetic solutes during freeze-thawing of LDH. HDB and HGB, which showed better stabilization compared to trehalose and GB during heat denaturation, were less effective during freeze-thawing. Even sulfobetaines were able to protect LDH effectively during freeze-thawing, and again SB-3 gave the best protection amongst the four sulfobetaines.

Freeze-drying often destabilizes proteins due to the conformational instability of many proteins when subjected to freezing and subsequent dehydration stresses (Crowe et al., 1990). In contrast to freeze-thawing, where freezing is the only stress, freeze-drying poses two stresses, freezing and drying. Thus, to protect proteins against freeze-drying, both these fundamentally different stresses have to be overcome. Several additives have been tried in order to protect proteins against freeze-drying, including disaccharides (Carpenter and Crowe, 1989) and compatible solutes (Lippert and Galinski, 1992; Galinski, 1993). Results presented here have shown that synthetic compensatory solutes have the potential to be used as stabilizers of proteins against freeze-drying.

Trehalose, which is known for its protection of proteins against desiccation stress, was the best stabilizer for both LDH and lipase. HDB was able to stabilize lipase very well, but had only a modest stabilizing effect on LDH. HGB was the most effective stabilizer of all the synthetic solutes. Most of the synthetic solutes with hydroxyl groups, except SB-1 and SB-3, were able to stabilize LDH during freeze-drying. It is suspected the impurities in these solutes may be the cause for their failure to protect LDH. Carpenter and Crowe (1989) proposed that hydrogen-bonding between the OH groups of the solute and polar residues of proteins is involved in maintaining the structure of the dried protein. This is consistent with the superior performance of synthetic solutes with OH groups. As expected, HGB with two OH groups in its structure is a better stabilizer than HDB and DB (one OH group). However, we are unable to explain why DEHB (with two OH groups) does not stabilize as effectively as HGB (see Appendix A for structures of compensatory solutes). The speculation in this case would be that DEHB is not pure enough and the impurities may be affecting the enzyme activity.

Lippert and Galinski (1992) showed that hydroxyectoine, a compatible solute containing OH group, was a superior stabilizer to other compatible solutes in their study of freeze-drying of LDH. Our results show that GB, propio betaine, dimethylthetin and CB-1, which do not have OH group in their structure, fail to protect LDH during freeze-drying, which is consistent with results of other investigators (Lippert and Galinski, 1992). Furthermore, these solutes (without OH groups) seem to destabilize the enzyme as the relative activity after freeze-drying in their presence is less than the no addition (Figure 4.4). Thus, our results further support the argument (Carpenter and Crowe, 1989) that OH groups in the solute structure are needed for hydrogen-bonding of the solute to the protein, which is necessary for the solute to preserve dried proteins. However, results with more proteins are needed for confident generalization.

When we compare, on a molar basis, the stabilization effect of trehalose and our synthetic solutes during freeze-drying of enzymes, trehalose is approximately twice the molecular weight of some of the synthetic stabilizers (e.g. HGB, HDB and DB), so there was only half the mass of synthetic solutes present (compared with trehalose) in the experiments.

Trehalose is expensive to purchase whereas some synthetic solutes are inexpensive to make, so on a gram basis synthetic solutes can be a cheaper option to stabilize proteins during freeze-drying.

Not many conclusions could be drawn from freeze-drying experiments using mixtures of compensatory solutes of as the effect of trehalose seemed to be dominant in every mixture. However, one useful result is that mixing HDB with trehalose (0.75 M HDB + 0.25 M trehalose) gave a similar stabilizing effect to 1 M trehalose. This may be a cost-effective method for freeze-drying proteins, as synthetic solutes, especially HDB, are very cheap to synthesize.

Results of this study indicate that compensatory solutes containing OH groups are better stabilizers of proteins. In a study, using *E.coli* as a model system, to evaluate a range of natural and synthetic betaines for their ability to protect against salt and urea stresses (Randall et al., 1998), it was observed that serine betaine and betonine (OH group containing solutes) were very good urea protectors, and they speculated it was a result of the hydrogen bonding capacity of the hydroxyl group. Hydroxyectoine has been proved to be a better stabilizer of enzymes compared to other compatible solutes (Lippert and Galinski, 1992). Even though our results support these findings, the stabilization effect of solutes with OH groups cannot be generalized. Except for HGB and HDB, which are consistent in stabilizing enzymes during heat denaturation, freeze-thawing and freeze-drying, there are other solutes such as DEHB and DB (also with OH group) which show a varying degree of protection with different enzymes and stress factors. Propio betaine, CB-1 and SB-1, were very good stabilizers during heat denaturation of LDH, but gave moderate protection to chymotrypsin and they also failed to protect LDH during freeze-drying. SB-3 (sulfobetaine containing a C₄ bridge between S and N in its structure) had a better stabilizing effect compared to SB-2 (sulfobetaine containing a C₃ bridge between S and N in its structure, see Appendix A) during heat denaturation of both LDH and chymotrypsin. However, during freeze-drying of LDH, SB-2 showed a good stabilizing effect and SB-3 failed to protect the enzyme. In a study of membrane-protein extraction (Vuillard et al., 1995), results have shown that sulfobetaines with a C₃ bridge between N

and S are more efficient than sulfobetaines with a C₄ bridge. Furthermore, they state that they were unable to find any application where C₄ bridge sulfobetaines would perform better than C₃ bridge sulfobetaines. Though our results of freeze-drying would support the above mentioned argument, our heat denaturation results show the contrary.

From our results of stabilization of different enzymes using a range of solutes and under different stresses, we believe that the stabilization effect of any particular solute cannot be generalized. The observed stabilization effect of a solute may be specific to the enzyme under investigation and the stress factor considered. These solutes may interact with each protein in a different way that would result in a different degree of stabilization. It is also possible that the properties of the solutes themselves may change in the face of different stresses, like high and low temperatures or salt stress, which would in turn affect the protein. Most of the proposed mechanisms of protein stabilization have considered only a few solutes or particular group of solutes to explain the mechanisms by which they stabilize the proteins. The new range of synthetic stabilizers that we have presented here may be helpful in providing new insights into the mechanism of protein stabilization as they are slightly different from each other in their structure and have varied degree of protection with different enzymes. Protein stabilization is a very important aim in the field of biotechnology and our results suggest that while deciding a stabilizer for a particular protein, it would be helpful to choose from a range of stabilizers instead of any one universal stabilizer.

Part B

4.2 Assessment of Stabilizing Effect of Compensatory Solutes on the Unfolding of Chymotrypsin Using an HPLC system.

In Part A of this chapter the stabilizing effect of synthetic compensatory solutes on catalytic activity of three different enzymes has been explored. These catalytic activity results do not explain much about the conformation of the enzyme/protein in the presence of compensatory solutes. Therefore, to understand more about the mechanism by which these solutes stabilize enzymes and their effect on protein conformation, the effect of compensatory solutes on unfolding of chymotrypsin using was investigated.

4.2.1 Introduction

Most of the enzyme stabilization studies have used activity measurements as the basis of their investigation. Only a few researchers (e.g. Goller and Galinski, 1999) have used fluorescence spectroscopy of endogenous tryptophan-residues as a technique for monitoring the structural changes of an enzyme. The fluorescence of tryptophan-residues depends on the conformation of the protein and usually changes with unfolding/denaturing of the native enzyme structure. As the enzyme unfolds, it exposes more tryptophan residues leading to changes in the fluorescence.

Goller and Galinski (1999) used time resolved fluorescence measurements to monitor the structural changes of LDH caused by freezing and thawing in the presence of compatible solutes. Their results revealed that residual fluorescence intensity correlated well with the residual activity determined by activity assays. They concluded that residual intensity reflects the proportion of native enzyme. In most of the studies involving stabilization of enzymes, the stabilization effect has been monitored with the help of enzyme activity measurements. However, in some of the studies inconsistencies have been observed between the stabilization of functional activity and the structure of the enzyme (Galinski, 1993; Burg et al., 1996; Goller and Galinski, 1999). To understand this, the effect of compensatory solutes/ co-solvents on the fluorescence of a number of probes has been

studied (Wood et al., 2000; Lever et al., 2001). 8-Anilinosulfonate (ANS) is one of the fluorescent probes that have been used in these studies. Randall et al. (1998) found that adding compensatory solutes to aqueous ANS solutions increased the fluorescence yield without a change in Stokes shift, and trehalose and glycine betaine increased the fluorescence most. However, their study had problems due to the contaminants in solutes which affected the fluorescence. The solutes used for these studies had to be pure, as any fluorescent impurities present would change the fluorescence being studied. This problem was overcome by Lever et al. (2001) by making their measurements using HPLC equipment. The HPLC degassing module and the reverse-phase column removed virtually all fluorescent impurities in the solutes. Results of changes in fluorescence intensity of ANS induced by co-solvents (Lever et al., 2001) showed that changes in fluorescence of these probes may throw light on the hydration of functional groups in larger molecules (proteins) and provide new insights into the stabilization of tertiary structure.

Chymotrypsin was chosen as the enzyme for our study. The emission maximum of denatured chymotrypsin is higher than that of its native form as the tryptophan-residues of chymotrypsin are exposed more in the denatured/unfolded state. This was used as the basis for our experiments. The emission maximum intensity of the protein usually changes in the presence of compensatory solutes and this change in the fluorescence can be related to the conformation of the protein. To ensure that the observed stabilizing effect of compensatory solutes is related to the conformation of chymotrypsin, we also assessed the effect of compensatory solutes on tryptophan solution and native/unheated chymotrypsin. An experimental set-up with HPLC equipment, similar to Lever et al. (2001), was used for our experiments. A reverse-phased HPLC column was used to remove the fluorescent impurities in the compensatory solutions. Degassed water was pumped slowly through an auto-injector and the column, and then mixed with the degassed aqueous solution of tryptophan or chymotrypsin in buffer, pumped by a second pump. The mixed solutions were passed through a coil to ensure proper mixing. For denaturation of the enzyme, the coil was maintained at a constant specified temperature. The fluorescence of the mixed solutions was measured simultaneously at two emission wavelengths (emission maxima corresponding to native and denatured states) using a fluorescence detector. To ensure that

the detector would record tryptophan or chymotrypsin in the solute solution for several minutes, a sufficient volume of each sample solute was injected.

4.2.2 Materials and Methods

4.2.2.1 Fluorescence Spectra

Fluorescence spectra of chymotrypsin (native and denatured) were obtained using a Cary Eclipse fluorescence spectrometer (Varian, Melbourne, Australia). Chymotrypsin solution (100 mg/L) prepared in 50mM sodium phosphate buffer, pH 8.0, was scanned and the fluorescence spectra of corresponding blank solution (buffer) subtracted. With the excitation wavelength being 290 nm, native chymotrypsin's maximum emission wavelength of 333 nm was determined by recording fluorescence emission spectra (300-400 nm) as the average of three measurements. To obtain the emission maxima of denatured chymotrypsin, the enzyme solution was incubated for 10 minutes at 50°C in a temperature block and scanned. The emission maximum of denatured chymotrypsin was found to be 358 nm.

4.2.2.2 HPLC system

Two HPLC pumps, Shimadzu LC-10 ADVP, were used for the experimental set-up (Figure 4.9). The pumps were referred to as 'A' and 'B'. Water passed through a Shimadzu DGU-14A membrane degassing module to Pump A (0.05 mL/min), and then through an auto-sampler, Shimadzu SIL10AXL fitted with a 400µL loop. The auto-sampler, controlled by a system controller (Shimadzu SCL-10A) contained 1 M compensatory solutions in 0.5 mL vials and was programmed to inject 400 µL to the sample stream. The sample stream then passed through a reverse phase column (Merck Superspher, RP 18 endcapped, 4µ, 250 × 2mm), which was maintained at a constant temperature of 50°C. All connecting tubing used in the system was 0.0007" i.d. PEEK HPLC tubing. Chymotrypsin (100 mg/L) in buffer solution (sodium phosphate, pH 8.0) or tryptophan solution (10 µM) in water,

passed through the degassing module to Pump B (0.05 mL/min) and through a back pressure regulator. The sample stream and chymotrypsin or tryptophan solution mixed at the “T” joint (Figure 4.9) and passed through a Teflon coil (1527 XL, Upchurch Scientific Inc., approx. 5 m, 0.010” id and 0.062” od) wound around an aluminum pipe of 1.6 cm outer diameter. The final concentration of the compensatory solutions was 0.5 M after mixing with tryptophan or chymotrypsin solution at the “T” joint. The approximate volume of the coil was 1 mL.

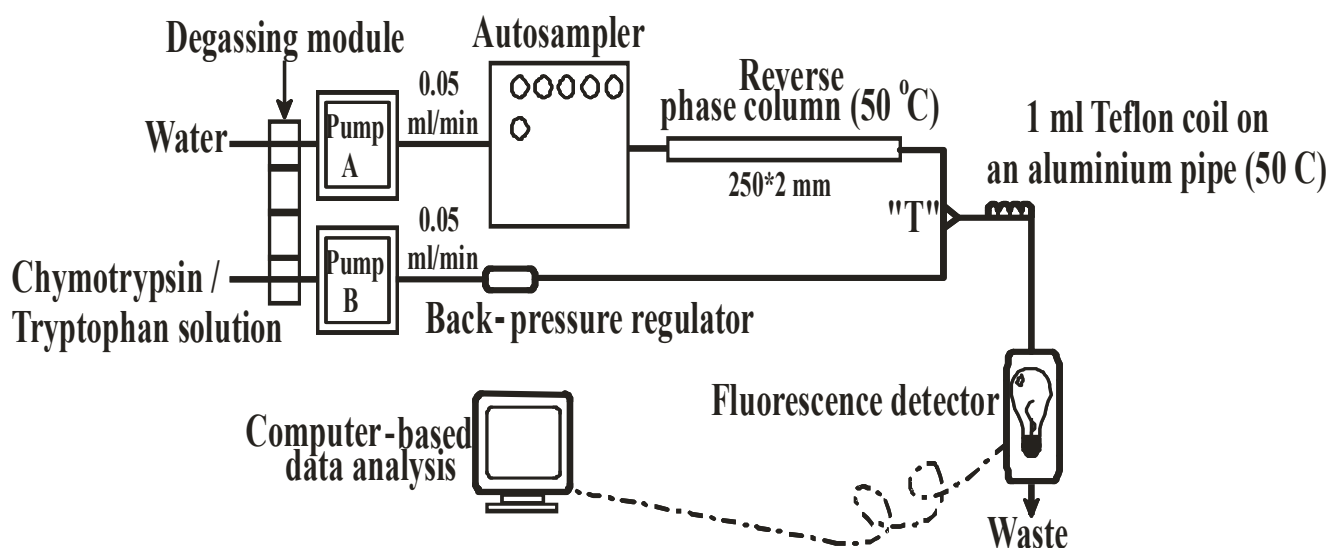


Figure 4.9: Experimental set-up. The arrangement used two Shimadzu pumps, a Shimadzu SIL10 autosampler fitted with a 400 μ L loop, and an Agilent 1100 series fluorescence detector.

The outlet from the coil was passed to a fluorescence detector, Agilent 1100 series connected to an Agilent 1100 hand held device. The fluorescence spectra of chymotrypsin showed that emission maxima of native and denatured chymotrypsin were 333 nm and 358 nm respectively. Hence, the detector was set at an excitation wavelength of 290 nm and fluorescence of the solution mixture (output from the coil) was monitored at two different emission wavelengths, 333 nm and 358 nm, simultaneously. These two emission wavelength outputs from the detector were monitored in two different channels using the DELTA Chromatography System (Digital solutions, Brisbane, Australia).

The Teflon coil was maintained at room temperature while monitoring the changes in fluorescence of tryptophan solution and unheated chymotrypsin in the presence of different compensatory solutes. However, during experiments to assess the stabilizing effect of compensatory solutes on denatured chymotrypsin, the coil was maintained at a constant temperature of 50°C, in order to partially denature the enzyme. Water was injected as a control to determine if there were any effects on the measured fluorescence due to pressure changes in the system during sample injection.

The base-line fluorescence for each run was the fluorescence of tryptophan or chymotrypsin solution measured at that particular wavelength, 333 nm and 358 nm, with water and no solute flowing through water stream. To determine the effect of each compensatory solute, the auto-sampler injected the compensatory solution into the water stream, which mixed with the tryptophan or chymotrypsin solution resulting in a sudden change in base-line fluorescence lasted for several minutes. This change in fluorescence was expressed as a percentage change in the base-line fluorescence. The percentage change in fluorescence ratio (Ratio % in Figure 4.11) was calculated from the plots of ratio of fluorescence at 333 nm and 358 nm in the presence of different solutes.

4.2.3 Results

As ion-exchange resin was used during the syntheses of DB and DMT, they were not used in the experiment due to the suspicion that impurities, if present, would affect the fluorescence. Propio betaine, DEHB and SB-1 were also not used as preliminary experiments using these solutes resulted in column blockage which in turn increased the pressure of the system. The concentration of solutes was 1 M during injection. However, after mixing with tryptophan/chymotrypsin solution their concentration was approximately 0.5 M at the detector. The fluorescence for each run was recorded for 60 minutes. The effect of injected compensatory solution on the fluorescence of tryptophan/chymotrypsin solution, both at 333 nm and 358 nm, appeared as a sudden change in the base-line

fluorescence between 16th and 18th minute and was usually a square flat-topped peak with a plateau width of 16 min (Figure 4.10). Other features can also be observed in Figure 4.10. There are a few peaks which appear within the first 10 minutes, and these are attributed to impurities in the solutes that were coming off the column.

Figure 4.10 A, B and C, show the detector output at the two different wavelengths acquired simultaneously. These figures show the changes in fluorescence observed when HGB was mixed with tryptophan, chymotrypsin and heated/denatured chymotrypsin solution. Figure 4.10D shows the plot of ratio of fluorescence at 333 nm and 358 nm of heated chymotrypsin in the presence of HGB and water. The changes in fluorescence of tryptophan, chymotrypsin and heated chymotrypsin solutions were expressed as % changes in the baseline fluorescence at that particular wavelength (Table 4.3). Table 4.3 also lists the change in fluorescence ratio for heated chymotrypsin, which is calculated from the plots of fluorescence ratio (333/358 nm).

At room temperature, the fluorescence of tryptophan solution (10 μ M), 333 nm and 358 nm, is minimally affected by any of the injected compensatory solutions. The percentage changes in fluorescence of tryptophan is slightly higher at 333 nm compared to 358 nm. SB-2, SB-3 and SB-4 increased the fluorescence of tryptophan more than the other solutes. In the case of unheated/native chymotrypsin (chymotrypsin at room temperature), the percentage changes in fluorescence were similar to that of tryptophan (Table 4.3). However, in plot 4.10(B) we observe that as the solute appears at the 16th minute, there is a sudden dip and a sudden increase in the fluorescence of chymotrypsin. We suspect this is due to the observed changes in the pressure of the HPLC system with the injection of the sample.

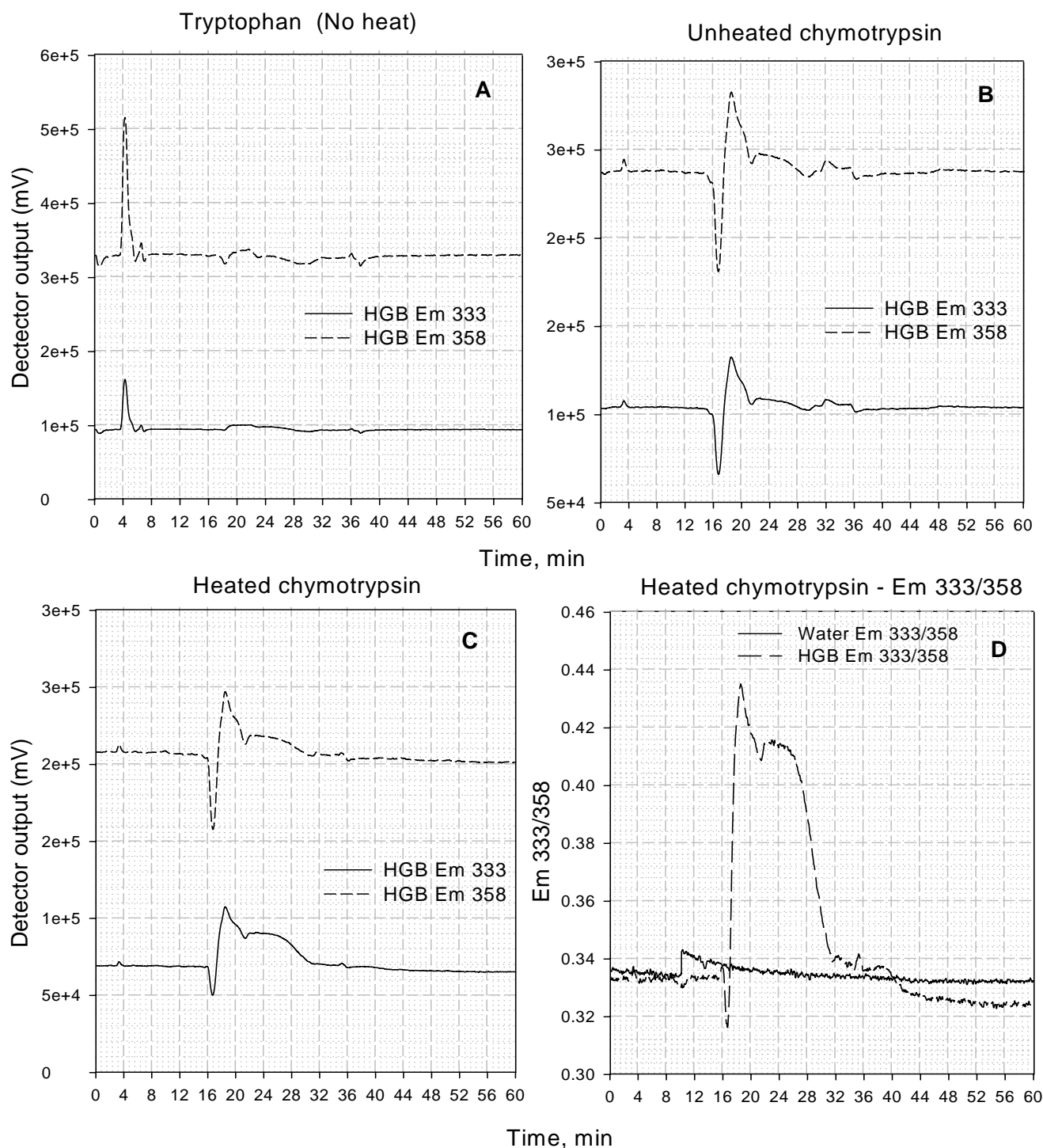


Figure 4.10: Plots showing the changes in fluorescence of tryptophan (10 μ M) and chymotrypsin solution (100 mg/L) at two different emission wavelengths, 333 nm and 358 nm, when 1 M homoglycerolbetaine (HGB) is injected. (A) Tryptophan solution without heat. (B) Chymotrypsin solution without heat. (C) Heated chymotrypsin solution (50°C). (D) Ratio (Em 333/358) of heated chymotrypsin (50°C) in the presence of HGB and distilled water. A higher ratio shows more native enzyme, i.e. less denaturation.

Table 4.3: Changes in fluorescence intensities of tryptophan and chymotrypsin solutions affected by presence of compensatory solutes

Cosolvent (second solute)	Changes in fluorescence in presence of 0.5 M cosolvent (% of baseline fluorescence)						
	Unheated Tryptophan (10 μ M)		Unheated Chymotrypsin		Heated Chymotrypsin (100 mg/L)		
All 0.5 M in detector	333 nm	358 nm	333 nm	358 nm	333 nm	358 nm	Ratio %
Glycine betaine	2.3	0.5	1.9	2.6	15.1	5.2	9.5
Sorbitol	3.2	2.7	2.7	3.7	11.0	4.3	6.3
Trehalose	3.6	3.0	3.1	3.4	12.9	5.1	8.0
Urea	0.7	0.6	1.8	1.9	-1.9	1.0	-2.3
HDB	4.2	-1.8	2.9	2.1	24.2	4.2	19.6
HGB	4.2	-1.2	4.7	3.8	31.8	6.2	24.7
CB-1	6.2	3.0	2.9	2.1	20.5	3.6	16.0
SB-2	6.3	4.7	6.8	6.3	23.1	7.0	16.0
SB-3	8.5	6.1	6.6	6.7	28.8	8.8	18.5
SB-4	4.2	1.8	4.8	4.2	19.0	5.3	13.5

Values quoted are percentage changes in fluorescence compared with that in pure water. The Ratio % is the percentage change in the ratio of the fluorescence at 333 nm to that at 358 nm at the same time point, calculated from plots of the ratio (i.e. Figure 4.11).

The few other changes observed in the plot (Figure 4.10B) are negligible if the Ratio % of changes in fluorescence at 333nm and 358 nm was considered. These results show that none of the compensatory solutes considered for these experiments show any effect on the native conformation of chymotrypsin at room temperature.

In the case of heated chymotrypsin (50°C), as the enzyme is denatured partially, significant changes in fluorescence are observed with the injection of compensatory solutes. The percentage change in fluorescence is more at 333 nm than at 358 nm (Table 4.3). At 333 nm, 10 – 15% increase in baseline fluorescence was observed in the presence of natural solutes, GB, trehalose and sorbitol. All the synthetic solutes significantly increased, between 20% and 32%, the fluorescence of denatured chymotrypsin at 333 nm. Urea, a denaturant, decreased the fluorescence at 333 nm. We also observed that the effect of compensatory solutes on the fluorescence of heated chymotrypsin at 358 nm is very minimal. However, compared to their effect on fluorescence of tryptophan and native chymotrypsin at 358 nm, the effect on heated chymotrypsin at 358 nm is more.

The measure of stabilizing effect of compensatory solutes on the unfolding of chymotrypsin was shown by the plots of ratio of fluorescence at 333 nm and 358 nm for heated chymotrypsin (Figure 4.11). The change in fluorescence ratio (Ratio %) calculated from these plots show the stabilization effect of individual solutes (Table 4.3). As these plots are the ratios of fluorescence at 333 nm and 358 nm, with 333 nm being the emission maximum of native chymotrypsin and 358 nm of denatured chymotrypsin, higher values of Ratio % indicate higher degree of stabilization. Figure 4.11 shows the flat-topped peaks with a plateau width of 16 min. The best stabilization effect is seen in the presence of HGB and HDB with their Ratio % values of 24.7% and 19.6%. Even sulfobetaines protected chymotrypsin against unfolding with their Ratio % changes between 16% and 19%. Natural solutes GB, trehalose and sorbitol, gave a moderate protection to chymotrypsin. Urea, as expected, being a denaturant seemed to unfold the denatured chymotrypsin further with its Ratio % change of -2.3%. The denaturation effect of urea is evident from the plot of ratio of fluorescence (Figure 4.11). As expected, no change in ratio of fluorescence is observed when pure water is injected.

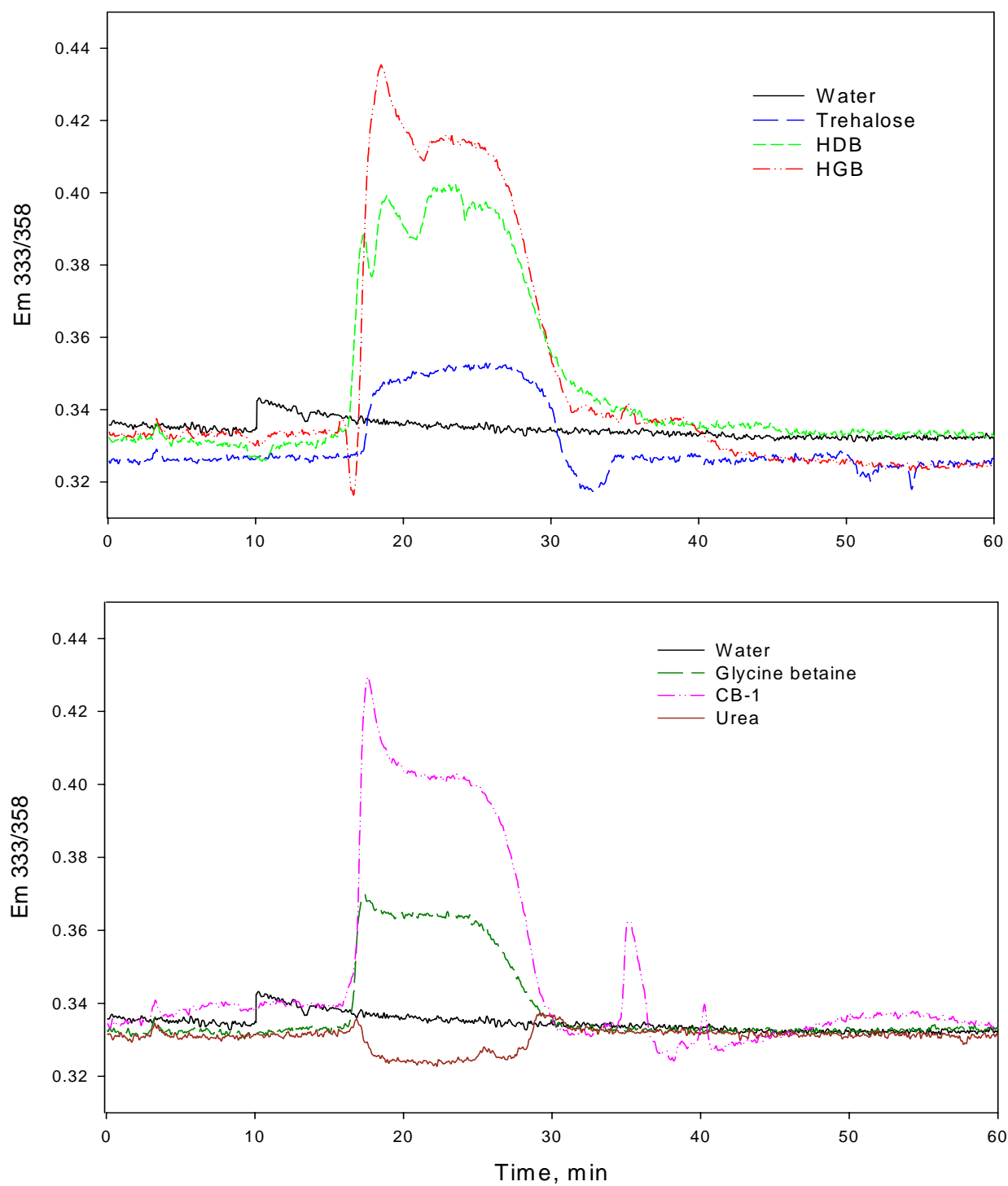


Figure 4.11: Ratio of fluorescence at 333 nm to that at 358 nm of heated chymotrypsin (50°C). The change in fluorescence with respect to baseline in each plot is due to the injection of 1 M compensatory solution.

4.2.4 Discussion

Fluorescence spectroscopy has been used before to monitor the structural changes of an enzyme during its denaturation (Goller and Galinski, 1999). The effects of compensatory solutes and other co-solvents on the fluorescence of a number of probes have also been studied (Lever et al., 2001). The experiment presented here, to our knowledge, is the first attempt to use fluorescence spectroscopy along with an HPLC system to assess the stabilizing effect of solutes on the unfolding of a protein. The fluorescence of tryptophan-residues of a protein mainly depends on the conformation of the protein and when a protein unfolds, it exposes more tryptophan residues to the environment leading to changes in the fluorescence. This was used as the basis of our experiments and investigated the effect of compensatory solutes on the unfolding of chymotrypsin.

As the emission maxima of native and denatured chymotrypsin are 333 nm and 358 nm respectively, the change in fluorescence was monitored at both these wavelength simultaneously. The reverse-phase HPLC column was mainly used to remove the fluorescent impurities from the sample stream. Alternative columns were also evaluated in order to check if we could improve the results. However, their ability to purify the samples was less. Impurities in solutes were suspected as the cause for blockage of column, observed during the use of propio betaine, DEHB, and SB-1.

To make sure that the results obtained relate to the structural changes of the protein, we also checked the effect of compensatory solutes on aqueous tryptophan solution (10 μ M) and unheated/native chymotrypsin. Results of our experiments clearly show that compensatory solutes do not affect the fluorescence of tryptophan or the native chymotrypsin. However, when the chymotrypsin is partially denatured by heating the solution at 50°C, a significant change in the fluorescence is observed. These results, plots of ratio of fluorescence (Figure 4.11) and ratio % change in fluorescence (Table 4.3), show the stabilizing effect of compensatory solutes. Synthetic solutes, HDB and HGB, were the best stabilizers and prevented the unfolding of chymotrypsin due to heat.

In Part A of this chapter, results of effect of compensatory solutes, natural and synthetic, on the activity of chymotrypsin against heat was presented. In Part B, results show the effects of solutes against unfolding of chymotrypsin due to heat stress. To understand if there was any relation between the stabilization effect observed between catalytic activity and conformation, T_D-50 values of chymotrypsin (Table 4.1) was plotted against Ratio % change in fluorescence (Table 4.3) in the presence of different solutes.

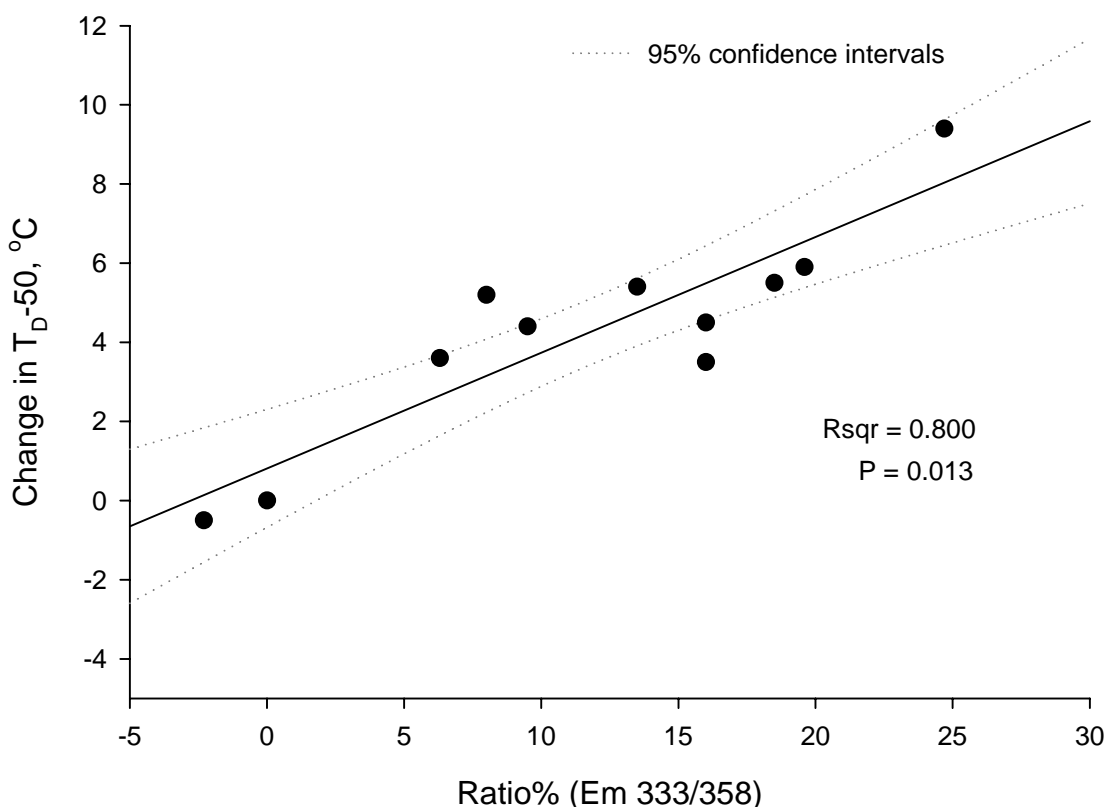


Figure 4.12: Simple regression plot of relation between Change in T_D-50 (Table 4.1) and Ratio % (Table 4.3) of chymotrypsin in the presence of different compensatory solutes. (Note: T_D-50 for chymotrypsin in the presence of 1 M urea was found to be $41.0 \pm 0.3^\circ\text{C}$)

Figure 4.12 clearly shows that there is a correlation between the stabilization effect observed on the catalytic activity and the conformation of the enzyme. It is evident that the stabilization effect of solutes which helped the enzyme retain its catalytic activity in face of severe stress, is not just on the catalytic activity but also on the conformation of the whole

enzyme. Though we can't generalize, these results are a good example of showing that good stabilizers protect the enzymes by preventing them from unfolding and helping them to retain their native structure.

Though the results give a clear picture about the stabilization effect of compensatory solutes on proteins, we have to keep in mind that our experiment was done using a limited number of solutes and on a single enzyme. It is suggested that further experiments, with various enzymes and more compensatory solutes, are needed to confirm that the results apply to other systems.

Part C

4.3 Study of Effect of Compensatory Solutes on Corticosteroid Binding Globulin Using an Enzyme-Linked Immunosorbent assay

In this part of the chapter, the study on the effect of compensatory solutes on a different model protein, corticosteroid binding globulin (CBG), is reported.

4.3.1 Introduction

Human corticosteroid-binding globulin (CBG), a circulating glycoprotein, is reported to bind 90-95 % of plasma cortisol with high affinity (Westphal, 1986). The bioavailability of endogenous glucocorticoids is predominantly influenced by CBG, and altered levels of CBG results in altered free cortisol levels (Coolens et al., 1987). Bonte et al. (1999) suggested that the free cortisol index could be calculated from total cortisol and CBG levels. CBG levels have been reported to be decreased in polycystic ovary syndrome in women (Invitti et al., 1991) and in a rare case of cyclical Cushing's disease (Watanobe et al., 1995).

As there is sufficient interest in CBG, researchers have tried to develop new methods, especially enzyme-linked immunosorbent assay (ELISA), to determine CBG levels in human plasma. A new “in house” ELISA to human CBG has been established at the Steroid and Immunochemistry Laboratory, Canterbury Health Laboratories, New Zealand (Lewis et al., 2003). This ELISA is a 2-site assay where CBG is captured by an immobilized polyclonal antibody to CBG that is subsequently detected by a CBG monoclonal antibody. This method has been thoroughly validated and compared against a ligand binding method.

Previous studies to understand the true quantitative role of CBG in binding circulating cortisol in human plasma (Lewis et al., 2005) used heat treatment of plasma as a method to inactivate CBG. Heat treatment of plasma at 60°C for 60 min led to total inactivation of

CBG. This inactivation was further confirmed by the undetectable CBG levels by ELISA. This inactivation of CBG with heat treatment was used as another model to study the stabilizing effects of synthetic compensatory solutes. Heat treatment of plasma was done in the presence of different compensatory solutes, and then CBG levels were measured by ELISA.

4.3.2 Materials and Methods

4.3.2.1 Materials

Human CBG, sheep antibody to CBG (IgG fraction) and antimouse IgG Fc- peroxidase were obtained from Cortex Biochem, San Leandro, CA, USA. Antimouse Ig-peroxidase was from Silenus Labs, Boronia, Victoria, AUS.

4.3.2.2 Heat treatment

Human plasma was mixed with equal volumes of 2 M compensatory solutions (final concentration: 1 M) and then heated in a temperature block for 75 min at 60°C. Aliquots of this mixture were taken at 0, 15, 30, 45, 60 and 75 min, and diluted (1:1000) in assay buffer for ELISA. As a control, plasma was mixed with equal volume of assay buffer and heated.

4.3.2.3 CBG ELISA

ELISA plates (Falcon 3912 microtest III; Beckton Dickinson, Oxnard, CA, U.S.A.) were coated with sheep anti-CBG serum diluted in PBS (30 µL in 10 mL PBS), 100 µL per well, and was left for overnight incubation at 4°C. Following coating the plates were washed and blocked with assay buffer (150 µL per well) for 1h at 20°C. The plates were emptied by inversion and either 100 µL of standard or patient plasma (1:1000 dilution in assay buffer) added to each well for an overnight incubation at 4°C. Standard plasma was pooled third trimester pregnancy plasma with a CBG level of 1280 nmol/L at 1:1000 dilution. However, for the standards this was diluted 1:500 in assay buffer and then serially to generate a series of standards from 0 to 2560 nmol/L. Following overnight incubation the plates were washed and supernatant 12G2 diluted (1:20 in assay buffer) 100 µL per well for 2h at 20°C. The plates were then washed and 100 µL of anti-mouse IgG Fc-peroxidase (1:1000

in assay buffer) added for 1 h at 20°C after which the plates were finally washed and substrate added, 100 μ L/well. Substrate was prepared by the addition of 600 mL aqueous solution containing 8.2 g anhydrous sodium acetate and 3.6 g citric acid to 400 mL of methanol containing 270 mg of tetramethyl benzidine. 500 μ L of 30 % H₂O₂ was finally added and the substrate stored in a dark bottle at room temperature. Colour development was terminated by the addition of 100 μ L of 1.25 M H₂SO₄ per well and absorbance was read at 450 nm on a BMG Fluostar Galaxy (BMG, Technologies GmbH, Germany). To avoid evaporation losses plates were covered for all steps preceding the addition of substrate.

CBG levels from the spectrophotometer were obtained as nmol/L. However, here we express CBG levels in the presence of different compensatory solutes and at different temperatures as relative CBG (%); relative to the CBG level found in presence of that particular solute at 0 min incubation (room temperature).

4.3.3 Results

Results of relative CBG in the presence of different compensatory solutes were plotted against time of incubation and are as shown in Figure 4.13. In the absence of any compensatory solutes (plasma heated with buffer), approximately 80 % of total CBG was inactivated after 15 minutes of incubation and was fully inactivated after 60 minutes heat treatment. Of all the compensatory solutes considered for this study, higher levels of CBG were found only in the presence of a few solutes. Best protection of CBG was found in the presence of HDB, DEHB and propio betaine. HGB was not able to confer any protection to CBG against heat. Natural stabilizers GB, sorbitol and trehalose showed moderate protection against heat inactivation of CBG. Sulfobetaines, SB-1, 2, 3 and 4, were not able to confer any protection to CBG. Error bars shown in Figure 4.13 are the standard error of means. The error in CBG measurement was observed in the presence of solutes that protected CBG against heat (e.g. HDB, DEHB, DB and propio betaine)

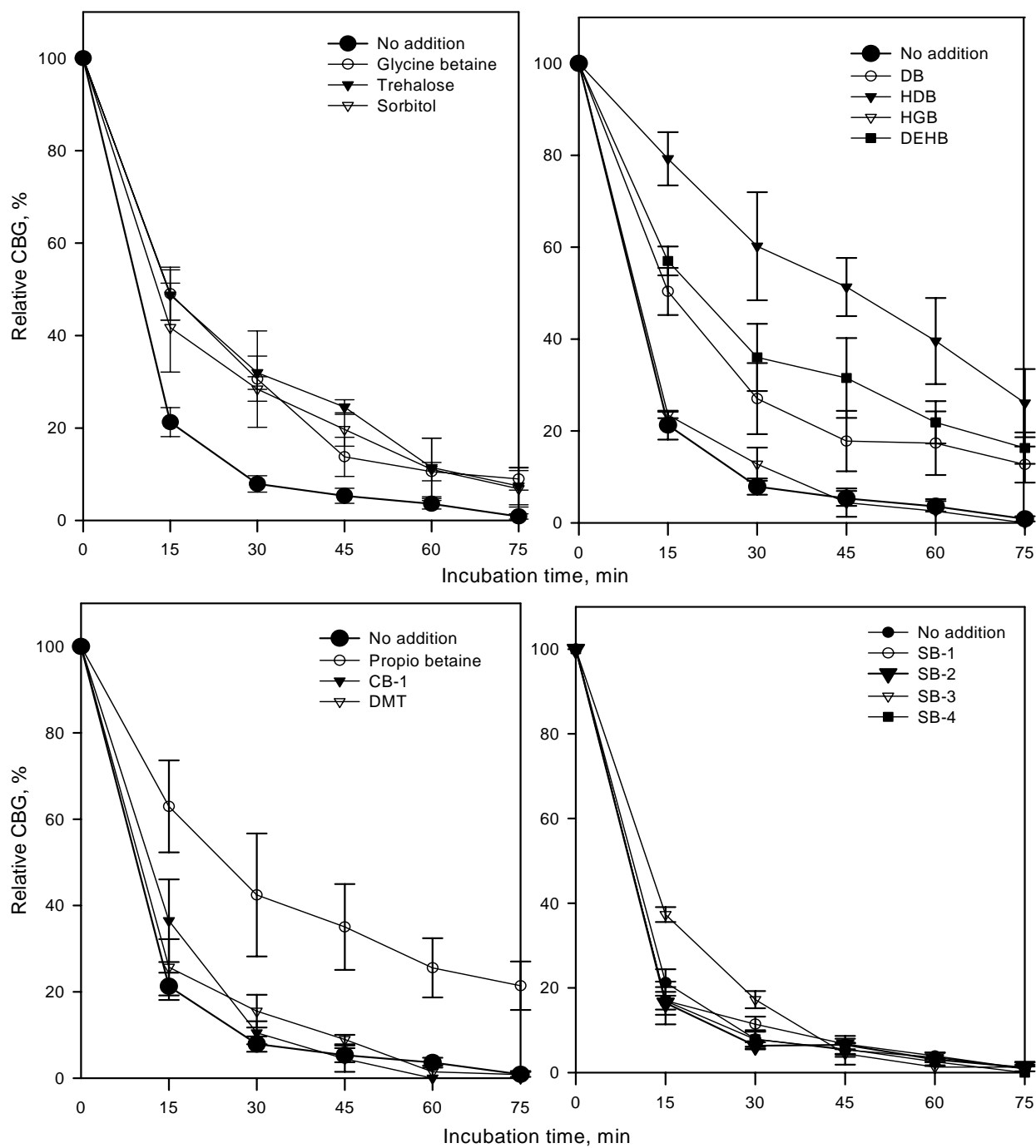


Figure 4.13: Relative CBG (%) \pm SEM in human plasma with heating at 60°C for 75 min in the presence of 1 M (final) compensatory solutions.

4.3.4 Discussion

CBG immunoreactivity is normally destroyed by heat treatment of plasma and a likely explanation for this is that the heat treatment of plasma normally masks the antigenic determinants due to partial denaturation of CBG (Lewis et al., 2003). Here we have investigated the effect of compensatory solutes against inactivation of CBG due to heat treatment.

From the results we are able to identify a few synthetic solutes such as HDB, DEHB and propiono betaine, which can protect CBG from inactivation even after 60 min of incubation at 60°C. Interestingly, HGB, which was a very good stabilizer of catalytic activity of LDH and chymotrypsin, and helped protect chymotrypsin against unfolding, was not able to prevent the inactivation of CBG. Even sulfobetaines, which were good stabilizers of native conformation of chymotrypsin in the face of heat stress, were not able to confer any kind of protection to CBG.

From the results we are able to show that relatively higher levels of CBG are found in plasma even after 60 minutes of incubation at 60°C in the presence of synthetic solutes HDB, DEHB and propio betaine. Though we are able to show some positive results from CBG experiments, one of the main problems faced during these experiments was the relatively large standard errors of the mean shown in Figure 4.13 when using test solutes compared to the control plasma without heat or solute. Several attempts and changes to the experiment were made to check if these errors could be overcome, but they did not improve.

As the CBG ELISA has been validated and has previously shown acceptable performance (Lewis et al., 2003), these errors could have arisen from mixing of intact plasma with very high concentrations of compensatory solutes (2M) and the increased possibility of differential heating due to variable viscosity compared to control plasma without solute. In addition, non-heated plasma controls with solute are also subject to variation, indicating that the standard ELISA is being affected by the presence of high concentrations of solutes,

and for this reason each solute was compared with its non-heated control and the results expressed accordingly. From Figure 4.13 it can be observed that the standard error of means is less in the case of ‘no addition’ (control), but high in the presence of good stabilizers such as HDB, DEHB and propio betaine. This shows that the standard ELISA is being affected by the presence of high concentrations of our additives.

Plasma also plays an important role in these experiments. Our experiments were done over a period of several months necessitating the use of several different lots of plasma. Normal plasma levels of CBG (nmol/L) are significantly different for males and females; 609 ± 242 and 743 ± 271 nmol/L respectively (Lewis et al., 2003). Decreased CBG levels have also been reported in humans suffering from different conditions (Bladon et al., 1996; Pugeat et al., 1989). These altered levels of CBG in different individuals may also be one of the reasons for the observed high standard errors in our results.

Notwithstanding these limitations the results clearly show that a few of our synthetic solutes can be used to prevent the inactivation of CBG due to heat. These results support observed stabilization of catalytic activity and conformation of proteins by synthetic compensatory solutes in sections 4.1 and 4.2 of this chapter.

4.4 References

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Chapter 5 - Medical Application of Betaine Analogues: Addition of Glycine Betaine or a Synthetic Analogue to Peritoneal Dialysis Fluid

5.1 INTRODUCTION

5.1.1 Kidney and its function

Blood performs the essential function of bringing nutrients and oxygen to the cells of the body and carrying the waste materials away from those cells. It follows therefore, that the chemical composition of the blood must be carefully controlled for it to be able to perform its function. The kidneys, a pair of organs located just behind the lining of the abdominal cavity (retroperitoneal), are responsible for filtering the blood to remove the waste products of metabolism, such as, urea, uric acid and creatinine while also regulating its ionic concentration. They help in retaining the essential chemical constituents the body requires, while simultaneously regulating body fluid volume.

The functional unit of the kidney is called the nephron, of which, there are approximately 1 million within each healthy adult kidney localized in both the cortex and medulla. The nephron is responsible for separating the various components of the blood through three basic mechanisms: filtration; resorption; and secretion. When the kidneys do not function properly (renal failure), toxic waste starts building up in the blood, requiring it to be filtered by artificial means. Renal failure also affects various biochemical reactions of the kidneys and results in high or low concentrations of various components.

5.1.2 Real Replacement Therapy- Artificial Dialysis

For patients suffering from renal failure, dialysis is a procedure that filters and purifies the blood, ridding the body of harmful wastes, extra salts and fluids, while maintaining the proper balance of chemicals such as potassium, sodium, and chloride. There are two

modalities of dialysis for the treatment of renal failure: haemodialysis (HD) and peritoneal dialysis (PD) (Figure 5.1).

Haemodialysis uses a dialyzer made up of a cellulose-membrane tube that is immersed in a large volume of salt solution with ionic concentrations near, or slightly lower, than the desired blood concentration. During treatment, blood is pumped from the patient via an intravenous line through the dialyzer, which filters out waste and extra fluid, before being returned to the patient, again through an intravenous line (Figure 5.1). The membrane tubing in the dialyzer has a molecular-weight cut-off that will allow most solutes in the blood to exchange across the tubing wall enabling the retention of larger components such as proteins and cells.

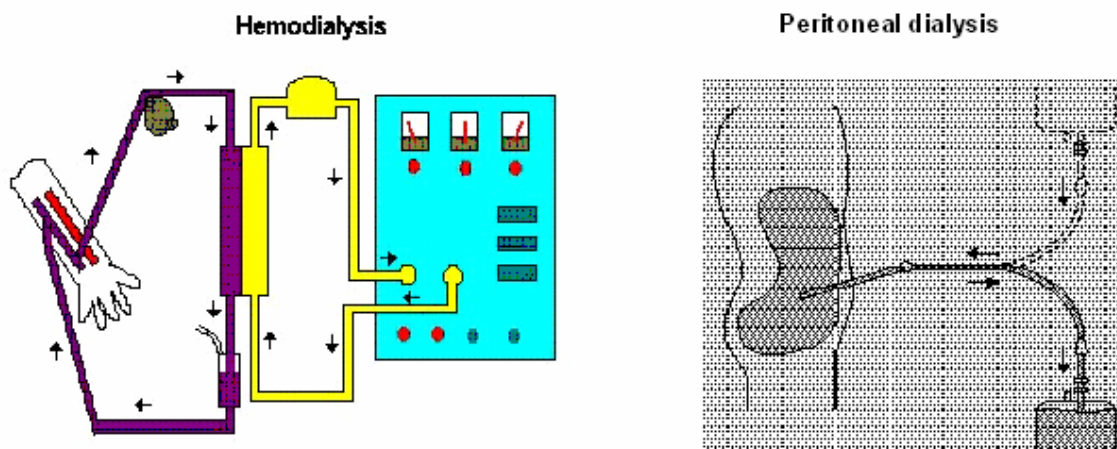


Figure 5.1: Diagrammatic representation of haemodialysis and peritoneal dialysis. (Haemodialysis (www.shodor.org) and peritoneal dialysis (Gokal & Mallick, 1999))

In peritoneal dialysis, the dialysis fluid flows into the abdomen via a catheter that has been surgically implanted. Once the fluid is inside the abdomen the catheter is closed and the fluid left to dwell for a pre-determined time (duration of dwell and fluid volume depends on an individual's dialysis prescription), after which, the fluid is drained out via the catheter. During peritoneal dialysis, solutes and fluids are exchanged between the peritoneal capillary blood and the dialysis solution in the peritoneal cavity across the peritoneal membrane. Laws of diffusion and convective transport govern the solute movement and the fluid exchange is related to osmosis created by the addition of

appropriate osmotic agents in the peritoneal dialysis solutions (Gokal and Mallick, 1999). Unlike haemodialysis, peritoneal dialysis is a passive process with fluid entering and leaving the abdomen via a gravity driven process. Thus it does not require the use of specialized machinery and patients can perform the fluid exchanges themselves. As the membrane cannot be manipulated in peritoneal dialysis, to achieve maximum solute and fluid removal, the flow rate of dialysis solutions and also the regime is changed frequently

5.1.3 Homocysteine (Hcy) and renal failure

Homocysteine (Hcy) is an intermediate in the metabolism of the essential amino acid methionine (Figure 5.2). Homocysteine is of interest clinically because elevated plasma total homocysteine (tHcy) concentrations in humans have been linked to an increased risk of vascular disease (cardio, cerebral and peripheral) (Boushey et al., 1995; Nygard et al., 1997; Welch & Loscalzo, 1998). Hyperhomocysteinemia is the condition which results when circulating tHcy is moderately elevated. Cardiovascular disease is a major cause of morbidity and mortality in patients with chronic renal failure (CRF) (Brown et al., 1994). Numerous studies have shown that more than 80% of chronic renal failure patients receiving dialysis treatment have elevated plasma tHcy (Bostom and Lathrop, 1997; Soria et al., 1990; Hultberg et al., 1993). The cause(s) of hyperhomocysteinemia in renal failure patients is unclear, however, the kidney primarily metabolizes Hcy rather than excreting it, and thus it is likely that hyperhomocysteinemia occurs in CRF patients as a result of disturbed Hcy metabolism.

Homocysteine is metabolized by one of two methionine conserving pathways (Figure 5.2), both of which involve the methylation of homocysteine to reform methionine. In a vitamin B₁₂ dependent reaction, homocysteine is methylated via the enzyme methionine synthase (E.C. 2.1.1.13) using *N*⁵-methyl-tetrahydrofolate as the methyl donor. The second pathway is catalyzed by betaine-homocysteine methyltransferase (BHMT- E.C. 2.1.1.5), where a methyl group is transferred from glycine betaine (*N,N,N*-trimethylglycine) to homocysteine, forming methionine and *N,N*-dimethylglycine.

BHMT is active in kidneys and liver of humans. Finkelstein and Martin (1984) estimated that the BHMT pathway contributes as much as 50% of the homocysteine methylation capacity of the liver. However, most research has focused on the methionine synthase methylation pathway, with most treatment strategies for the treatment of hyperhomocysteinemia involving folate and vitamin B₁₂ supplementation. Comparatively little attention has been placed on the BHMT remethylation pathway, or indeed on the regulation of glycine betaine homeostasis.

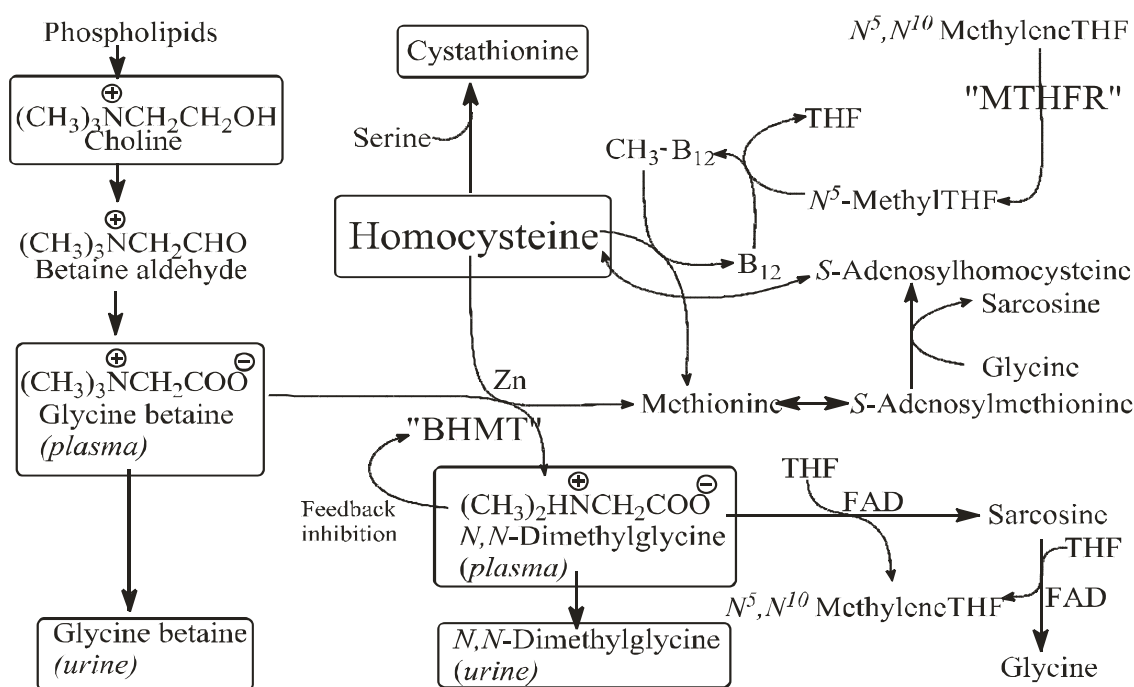


Figure 5.2: Homocysteine metabolism.

Glycine betaine (GB) is formed by the endogenous oxidation of choline (Flower et al., 1972) and is also obtained from the diet (de Zwart et al., 2003). It has been found that plasma concentration of GB is lower in patients with renal disease, and in non-dialysis chronic renal failure, renal clearance of GB is increased (Lever et al., 1994). Thus, replacing GB with alternative substrates for BHMT might help in regulating the Hcy metabolism.

In addition, one of the products of BHMT pathway, *N,N*-dimethylglycine, is found to be a potent feedback inhibitor of BHMT (McKeever et al., 1991; Millan & Garrow, 1998). This feedback inhibition may be one of the causes for disturbed Hcy metabolism leading to elevated Hcy concentrations.

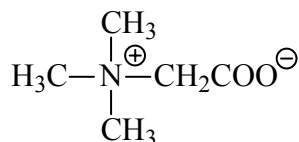
5.1.4 Therapies for Hyperhomocysteinemia

There have been several studies investigating various therapeutic treatments for reducing tHcy in end-stage renal failure patients in an effort to reduce the incidence of vascular disease. Some of the suggested treatments include: 1) medication via an intermittent intravenous regime, providing drugs containing low concentrations of folinic acid and vitamin B₁₂ (Buccianti et al., 2001), 2) long-term oral supplementation with either folic acid or folinic acid, in combination with high-dose intravenous vitamin B₆ and oral vitamin B₁₂ supplementation (Ducloux et al., 2002). These studies do not demonstrate the effect of these treatments on the patient and Ducloux et al. (2002) have suggested that the reduction of tHcy clearance may be due to multiple abnormalities of homocysteine methylation that may not be related to folate alone.

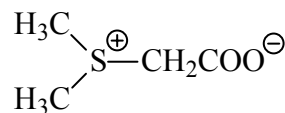
An alternative treatment strategy would be to increase Hcy methylation via the BHMT-mediated pathway by supplementing with GB. Oral GB supplementation has been shown to successfully reduce circulating tHcy concentrations in hyperhomocysteinemic patients (Wilcken et al., 1983), in non-dialysis CRF patients following a methionine load (McGregor et al., 2002), and in healthy volunteers (Olthof, et al., 2003). Nonetheless, the effect of GB, or various GB analogues, on homocysteine metabolism *in vivo* has not been studied extensively. Slow et al., (2004), showed in rats, that homeostasis of GB and Hcy are linked, and can be disturbed by both naturally occurring and synthetic GB analogues.

Dimethylthetin (DMT), a synthetic analogue of GB may be a potential therapeutic agent for lowering tHcy. Purified BHMT is shown to utilize DMT as a substrate at thirty-times the rate of GB (Goeger & Ganther, 1993) and it also displays greater specificity (V_{\max}/K_m) for BHMT reaction than betaine. Furthermore, the demethylated product of

DMT, methylthioacetate, does not inhibit BHMT as does *N,N*-dimethylglycine (Garrow, 1997). Slow et al. (2004) showed that a single dose of DMT was more effective for lowering tHcy when compared to an equivalent dose of GB, thus suggesting that DMT could be used as a therapeutic homocysteine-lowering agent.



Glycine betaine [GB]



Dimethylthetin [DMT]

5.1.5 Objectives

I was part of the team that had the objective to determine whether the addition of GB or DMT to dialysis fluid could relieve the chronic methyl donor shortage, increase homocysteine methylation via the BHMT-mediated pathway and lower circulating tHcy concentrations in CRF patients receiving dialysis. We chose peritoneal dialysis as the dialysis modality because it involves manual fluid exchanges without requiring the use of specialized dialyzing equipment. My role in this project was work with Dr. Sandy Slow in all the experimental work and to carry out the animal trials. We set out with three main aims:

- To develop an acute renal failure continuous ambulatory peritoneal dialysis (CAPD) model in sheep, this would be a true dialysis model that could better mimic the human clinical situation.
- To assess the effect on circulating homocysteine concentrations when DMT is added to dialysis fluid in a CAPD sheep model, using animals that were either in acute renal failure or had normal renal function.
- To assess the effect of addition of GB to dialysis fluid on homocysteine concentrations using the CAPD sheep model.

5.2 MATERIALS AND METHODS

5.2.1 The CAPD sheep model

5.2.1.1 Animals

The experimental protocol was approved by the Lincoln University Animal Ethics Committee in compliance with the New Zealand Animal Welfare Act 1999. Coopworth sheep (wether or ewe hoggets), weighing 33.5-52.2 kg, were housed indoors in individual metabolism cages for the duration of the experimental period. Animals were offered 1500g of food consisting of a mixture of high quality lucerne chaff, crushed barley and all-purpose ruminant pellets (Western Animal Nutrition, Rangiora, New Zealand, containing 85 % dry matter of which 16 % was crude protein and balanced micronutrients) and 4 L of tap water, per day. The animals were allowed to adjust to the diet and indoor environment for 1-2 weeks prior to commencement of the study.

5.2.1.2 Surgery

Two surgical operations were performed on each of the acute renal failure animals and one operation was performed on each of the animals with intact renal function. For all surgical procedures appropriate anesthesia (0.65 mg/kg diazepam (Pamlin[®], Parnell Laboratories, Auckland, NZ) and 13 mg/kg ketamine hydrochloride (Phoenix[®], Phoenix Pharmaceutical Distributors, Auckland, NZ) given intravenously and topped up if necessary) and analgesia (0.3 mg buprenorphine hydrochloride (Temgesic[®], Reckitt Benckimer Healthcare Ltd., Auckland, NZ, given intramuscularly as required) was employed.

The first operation, performed by Drs Robin McFarlane and Graham Barrell, was to insert a standard (47 cm universal), double-cuff Tenckhoff[®] peritoneal dialysis silicone rubber catheter (Tyco Healthcare Group, Mansfield, MA, USA), into the peritoneum to allow dialysis (Figure 5.3A). For 10-14 days following surgery, the catheters were flushed daily using 1 L of standard peritoneal dialysis fluid (either 1.36%, 2.27% or 3.86% glucose, Dianeal[®], Baxter, IL, USA) to ensure the catheter allowed flow in both directions. Before capping after the flush, the catheter was filled with a concentrated

heparin (10,000 IU; Leo Pharmaceutical Products, Ballerup, Denmark) and corticosteroid (20 mg methylprednisolone; Depo-Medrol[®], Pharmacia, NSW, Australia) solution to ensure patency.

The second operation, performed by veterinary surgeon, Dr. Larry Anderson, was a full bilateral nephrectomy to induce acute renal failure. The nephrectomy technique was based on that described by Horney and Archibald, (1961) and was performed at least 10 days after placement of the Tenckhoff[®] catheter. Following induction of anesthesia, a 20 cm vertical paracostal incision was made starting approximately 5 cm below the transverse processes in the para lumbar fossa. The skin was retracted and the underlying muscle layers were blunt dissected in a grid pattern to skirt around the peritoneum (retroperitoneal) and expose the right kidney. Perirenal adipose and connective tissue was removed from around the pedicle and the renal artery, renal vein and ureter were triple-clamped as one unit. The pedicle vessels were ligated using synthetic monofilament suture material before severing the vessels above the ligature to remove the kidney. The more mobile left kidney was located transperitoneally through the same incision site and removed following the same procedure as the right kidney.

To reduce the risk of post-operative infection, a mixture of penicillin and streptomycin (Penstrep LA, Bomac Laboratories Limited, Manakau City, NZ) was administered intramuscularly following each surgical procedure. Animals were denied food and water for one day prior to each operation and following the nephrectomy water availability was restricted to 1L per day.

5.2.1.3 Dialysis

Sheep 167, 174, 75 & 73 were used for the development of the acute renal failure CAPD model. At any one time, two animals went through the procedure and dialysis commenced within two hours of recovery from nephrectomy surgery. A standard human dialysis regime consisting of 4 exchanges per 24 hours using either 1.5 L (animal 167) or 2 L (animals 174, 75 and 73) of dialysis fluid per exchange was employed for up to 14 days.

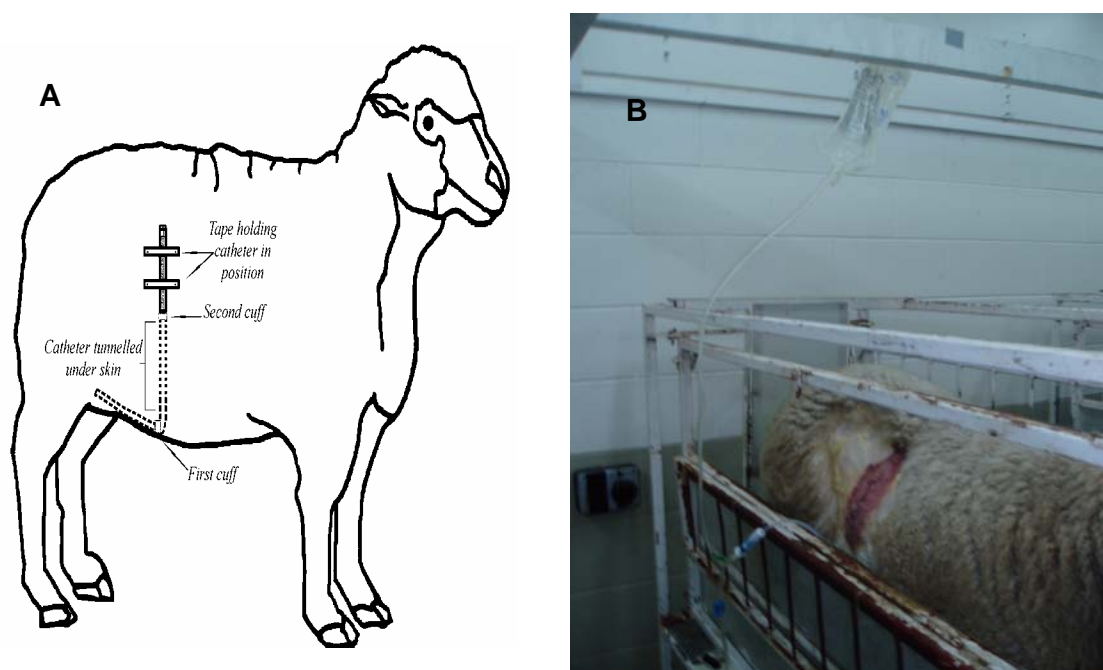


Figure 5.3: (A) Animal model showing the Tenckhoff[®] catheter position (B) Picture of a sheep undergoing dialysis showing the nephrectomy surgical site and the catheter.

In each 24-hour period, the first two animals (167 and 174) had a dialysis regime where the first 3 exchanges dwelled for 4 hours each and the final (4th) daily exchange dwelled for 12 hours (overnight). The glucose concentration of the dialysis fluid (Dianeal[®]) varied between exchanges, with concentrations of 1.36%, 2.27%, or 3.86% glucose being used, although 2.27% was the most common. However, as a result of fluid loading problems with the first animals (167 and 174) the regime was modified for the latter two animals (75 and 73) in which all exchanges had 3 hour dwells of 3.86% glucose (Dianeal[®]) dialysis fluid and without an overnight dwell. All dialysis fluid was warmed to body temperature before administration and following the last exchange each day, the catheter was filled with a concentrated heparin and corticosteroid solution to ensure patency.

5.2.2 Dialysis treatment using dimethylthetin (DMT) containing fluid

DMT was synthesized as described in section 3.2.1 of Chapter 3. Sheep 162, 163, 164 and 166, were used to study the effect of addition of DMT to dialysis fluid on plasma homocysteine concentration in three animals that were in acute renal failure (162, 163 & 166) and another that had intact renal function (164). Animals were housed indoors and surgery was performed as described above (5.2.1.2). Two animals went through the whole procedure at any one time.

Dialysis commenced within 2 hours of recovery from nephrectomy surgery, for animals 162, 163 & 166 and after the two week healing period following catheter insertion for animal 164. Animals 166 & 163 were given standard dialysis fluid containing 10g of DMT at each exchange, while animal 162 was maintained on standard dialysis fluid for 5 days, after which, 10g of DMT was added to the dialysis fluid for each exchange (Table 5.1). Animal 164 was given standard dialysis fluid containing 5g DMT per exchange (Table 5.1). All animals received 2L of dialysis fluid at each exchange and the DMT was added after the fluid had been warmed to body temperature via the injection port. Each day following the administration of the dialysis fluid for the fourth exchange, a concentrated heparin (10,000 U) and corticosteroid (20mg) solution was administered through the catheter to ensure patency.

5.2.3 Dialysis treatment using glycine betaine (GB) containing fluid

Glycine betaine was purchased from Sigma Chemical Co., USA. Sheep 65, 31 and 32, were used for this study. All animals were housed and maintained as described in section 5.2.1.2. Acute renal failure was induced in animals 65 and 32 following full bilateral nephrectomy, while animal 31 had intact renal function

Peritoneal dialysis commenced within two hours of recovery from nephrectomy for animals 65 and 32, and simultaneously for sheep 31. All the animals were given standard dialysis fluid containing 5g of glycine betaine per exchange. To avoid fluid loading problems all exchanges had 3 hour dwells of 3.86% glucose (Dianeal[®]) dialysis fluid and

without an overnight dwell. All dialysis fluid was warmed to body temperature, after which, glycine betaine was added to the fluid via the injection port before administration. After the last exchange (4th) each day, the catheter was filled with a concentrated heparin and corticosteroid.

5.2.4 Measurements and Assays

Blood samples were collected from each animal daily, both pre and post dialysis, via jugular vein venipuncture (5mL heparin and 5mL EDTA vacutainer; Becton Dickinson, Plymouth, UK). An aliquot of whole blood was removed for haematocrit measurement and plasma was separated from the remaining blood by centrifugation at 2000 g for 10 minutes. Plasma samples were sent to the High Volume Biochemistry Unit at Canterbury Health Laboratories, Christchurch, where all measurements were carried out using an automated analyzer (Aeroset Analyser, Abbott Laboratories, IL, USA). Routine biochemistry analyses (electrolytes, creatinine, urea, albumin and liver enzyme activity) and Hcy measurements were conducted on all samples. The heart rate, respiratory rate, rectal temperatures and behaviour of all animals were also monitored daily. The volume of the dialysate and its macroscopic appearance was recorded at each exchange for all animals, and if the dialysate had a cloudy appearance, an aliquot was sent for microbiological analysis (Microbiology Department, Canterbury Health Laboratories, Christchurch) to detect any infection. Animals were maintained on dialysis for up to 14 days post nephrectomy, after which, animals were euthanased by an intravenous administration of sodium pentobarbitone (overdose) followed by exsanguination. Post mortem examinations were conducted on all animals.

Plasma DMT and GB were measured by Dr. Sandy Slow using a HPLC method previously described by Lever et al. (1992). Martin Lee from Canterbury Health Laboratories assayed the dialysis fluid samples for methylthioacetate (the expected demethylated product of DMT) by ¹H-NMR as previously described (Slow et al., 2004).

5.2.5 Histopathological analyses

Samples of heart, brain, lung, and liver were collected at post mortem for all the animals and in addition, kidney samples were collected from animal 164, and sent to the Department of Pathology, Christchurch School of Medicine, for histopathological analyses. Tissue samples were fixed in 10% buffered formalin solution and embedded in paraffin. Tissue sections (3 μ m thick) were stained with haematoxylin and eosin and lung tissue sections were also stained using the periodic acid-Schiff reaction.

5.3 RESULTS

5.3.1 CAPD sheep model

One of the main problems faced while developing the sheep model was maintaining the catheter patency. In an earlier pilot study using sheep (Slow, S., personal communication; unpublished data), catheter patency was difficult to maintain. The catheters became blocked by proteinaceous clots and were persistently encased in omentum preventing fluid flow, in one or both directions. In an effort to avoid this, the catheters were flushed daily with 1L of dialysis fluid, after which, the catheters were filled with a concentrated heparin and corticosteroid solution. This regime proved to be successful for maintaining catheter patency and was adopted as standard practice throughout the dialysis studies described herein. One animal (174) had dialysis fluid leak into subcutaneous tissues post nephrectomy, a result of one of the catheter cuffs not adhering sufficiently to the skin. As a result effective dialysis could not be conducted and the animal was euthanased 24 hours following nephrectomy. All of the catheters in the other animals remained functional throughout the dialysis period. Of the three sheep that had functional catheters enabling adequate dialysis, animal 73 died on the 13th day post nephrectomy and animals 167 and 75 survived for the whole 14 day experimental period.

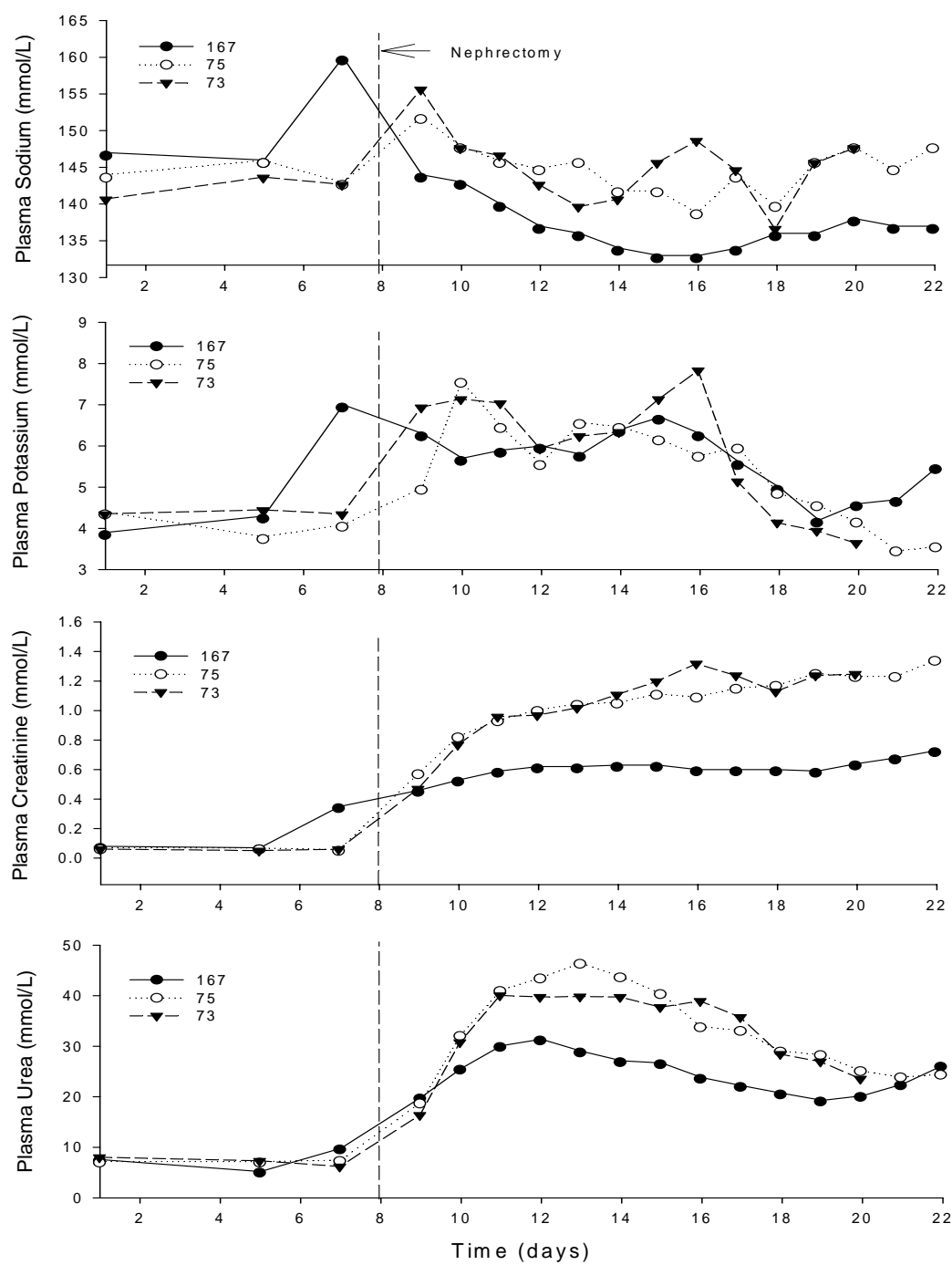


Figure 5.4: Daily plasma sodium, potassium, creatinine and urea of sheep numbered 167, 75 and 73 of CAPD sheep model.

Just after 24 hours post nephrectomy, the feed intake of all the animals decreased to approximately one third of pre-nephrectomy levels. Thereafter, the feed intake continued to decline and within 4-6 days post-nephrectomy all the animals had ceased eating. Although water was restricted to 1L per day, animals stopped drinking within the first 7 days of nephrectomy. As time on dialysis increased the animals became increasingly lethargic and non-responsive. In general, their heart rate increased and respiration rate slowed.

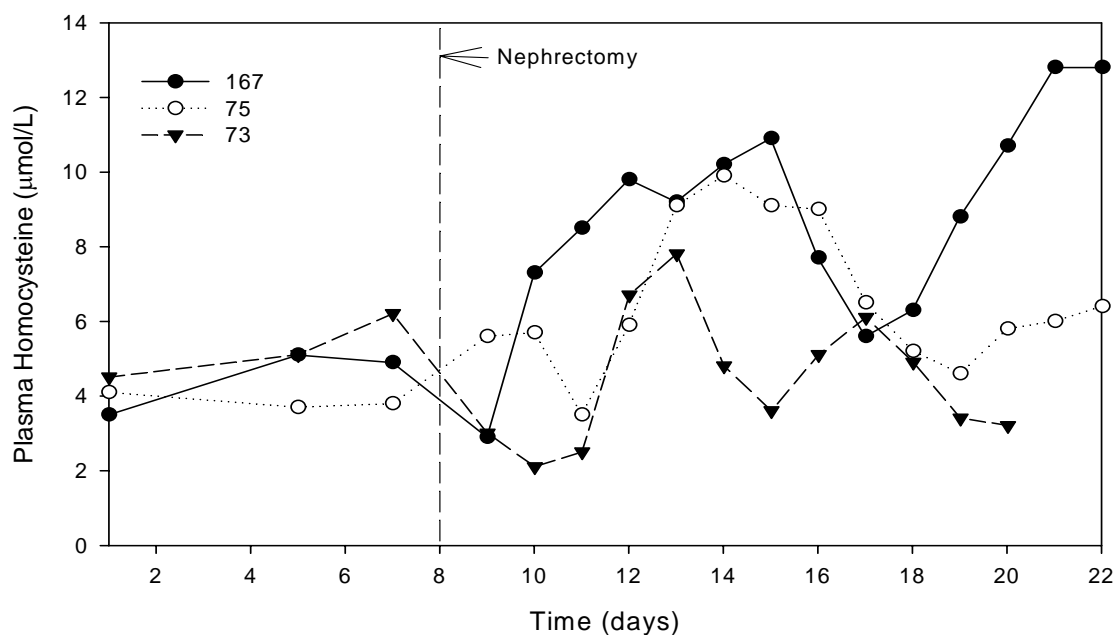


Figure 5.5: Daily plasma Hcy concentration for the three sheep on CAPD

During the first two days post-nephrectomy, plasma sodium and potassium concentration became elevated and reached maximum levels, but gradually decreased and returned to normal levels within 9 days after nephrectomy. Plasma calcium and magnesium concentrations fell rapidly post-nephrectomy and were normalized by addition of a supplement (50 mL of Glucalpos[®], Bomac laboratories limited, Manakau City, NZ) to the dialysis fluid. Haematocrit values were lower post-nephrectomy and declined as time on dialysis increased. Plasma creatinine concentrations increased throughout the dialysis period. Plasma urea concentrations increased significantly immediately following

nephrectomy and continued to increase, reaching maximum levels 5 days after the start of dialysis treatment, then they declined, but remained above normal concentrations.

Plasma Hcy concentration increased for all the three animals (167, 75 and 73), and remained higher than the pre-nephrectomy levels throughout the dialysis period. In animal 167, plasma Hcy concentration was approximately three times its pre-nephrectomy levels at the end of 14 day dialysis period (Figure 5.5).

5.3.2 Dialysis with DMT containing fluid

Initially, only two nephrectomised animals (163 & 166) were given dialysis fluid containing DMT (10g/exchange) and the other nephrectomised animal (162) was given standard dialysis fluid (Table 5.1). Unexpectedly, however, the animals that were being treated with DMT began to show adverse affects within 48 hours of beginning treatment. It was not known whether the symptoms that were observed were a result of the trauma of nephrectomy or because of possible DMT toxicity. It was decided after maintaining animal 162 on dialysis for 5 days with standard fluid, to start giving fluid containing DMT to see if the same effects were observed. In addition, we began dialysis on a non-nephrectomised animal (164) using standard fluid containing only 5g DMT per exchange to determine whether renal function was required for DMT treatment and whether giving less DMT per exchange had a similar effect.

Sub-acute exposure to DMT was found to be toxic in all animals, regardless of renal function or dose per exchange (10g or 5g). Although there was individual variation in the number of doses before adverse side affects were observed, generally the animals treated with DMT died within 48 hours of beginning treatment, averaging less than 7 doses (Table 5.1). DMT was effectively taken up from the dialysis fluid into the circulation and continued to increase depending on the number of doses each animal was exposed to. DMT was not detected in pre-dialysis plasma samples, and the final concentration at death ranged from 0.5-7.8 mmol/L (Table 5.1). The expected de-methylated product of

DMT, methylthioacetate was not detected in plasma or dialysis fluid, nor was methylthioacetate sulfoxide.

Symptoms of DMT toxicity included an increased heart rate, laboured breathing and excessive salivation from the nose and mouth. Animals became progressively non-responsive, lethargic and ceased eating and drinking. Even if DMT treatment was stopped after the animal exhibited initial respiratory difficulties (166, final four exchanges were with standard dialysis fluid only), the animal did not recover, continuing to decline until it was euthanased when the severity of the symptoms exceeded ethical limits.

Table 5.1

Sheep Number	Nephrectomy	DMT dose /exchange (g)	Number of DMT doses	Time on dialysis with DMT treatment (hours)	Plasma DMT concentration at death (mmol/L)
162*	Yes	10	8	40	8.2
163	Yes	10	4	24	1.4
164	No	5	7	48	0.5
166†	Yes	10	6	60	7.8

* Animal spent 5 days on dialysis with standard fluid, after animals 163 and 166 showed signs of possible DMT toxicity, this animal was switched to fluid containing DMT.

† DMT treatment ceased once animal exhibited initial respiratory difficulties, the final four dialysis exchanges were done using standard dialysis fluid.

At post mortem, compared to other animals treated with standard dialysis fluid only, the lungs of the DMT treated animals were abnormal in colour (deep red/purple) and were consolidated and heavy. The lungs did not float when placed in water and the animals appeared to have died from lung failure. No other organs seemed to have been affected, with the liver, brain, heart and kidney having a normal appearance. Histologically, lung tissue from DMT treated animals (Figure 5.6 A) when compared to lung tissue from

animals treated with standard dialysis fluid (Figure 5.6 B) showed complete lung consolidation and collapse.

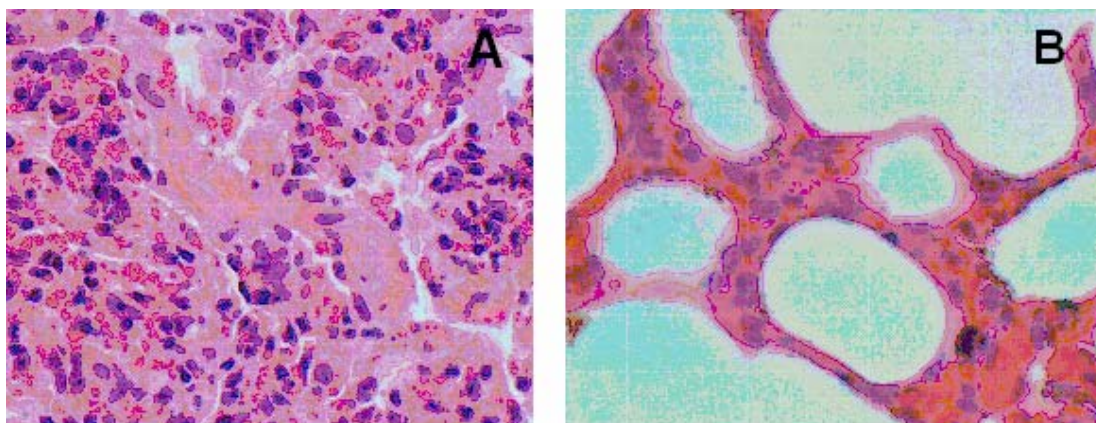


Figure 5.6: Sheep lung tissue sections (A) Nephrectomised animal treated with dialysis fluid containing dimethylthetin (DMT) (B) Nephrectomised animal treated with just standard dialysis fluid.

5.3.3 Dialysis with GB containing fluid

The dialysis regime was started simultaneously for two nephrectomised animals (65 & 32) and one non-nephrectomised animal (31). All three animals were given dialysis fluid containing GB (5g/exchange). No adverse or toxic effects were observed in any of the animals. Animal 32 developed a blockage in the catheter after eight days on dialysis treatment with no out-flow of the dialysis fluid. As a result, the animal had fluid build-up in the peritoneum and dialysis had to be discontinued and the animal euthanased.

After 12 days of dialysis, the non-nephrectomised animal (31) developed a blocked catheter and the dialysis was stopped. However, as the kidneys of the animal were intact, the animal was not euthanased. Animal 65 survived the full 14-day dialysis period with GB treatment and was euthanased on the 15th day.

The plasma Hcy concentration of the nephrectomised sheep (65 & 32) appeared to increase following nephrectomy, even with GB treatment (5g/exchange). Animal 32 was euthanased after 7 days on dialysis, and as a result it is difficult to draw any significant conclusions from the results obtained. Animal 65 showed similar changes in Hcy concentration as the CAPD model animals (167, 73 and 75). However, there was a trend towards lower Hcy during the last few days of dialysis (Figure 5.7). Plasma Hcy concentration in animal 31 (kidneys intact) appeared to decrease following the start of dialysis with GB containing fluid and was less than half the starting concentration at the end of dialysis period (Figure 5.7).

At post mortem, the lungs of the GB treated animals had no abnormalities in colour with a normal appearance. None of the other organs, liver, brain, heart and kidney, appeared to have been affected by GB treatment. The lung tissue samples from the GB treated animals were similar to animals treated with standard dialysis fluid (Figure 5.6(B)).

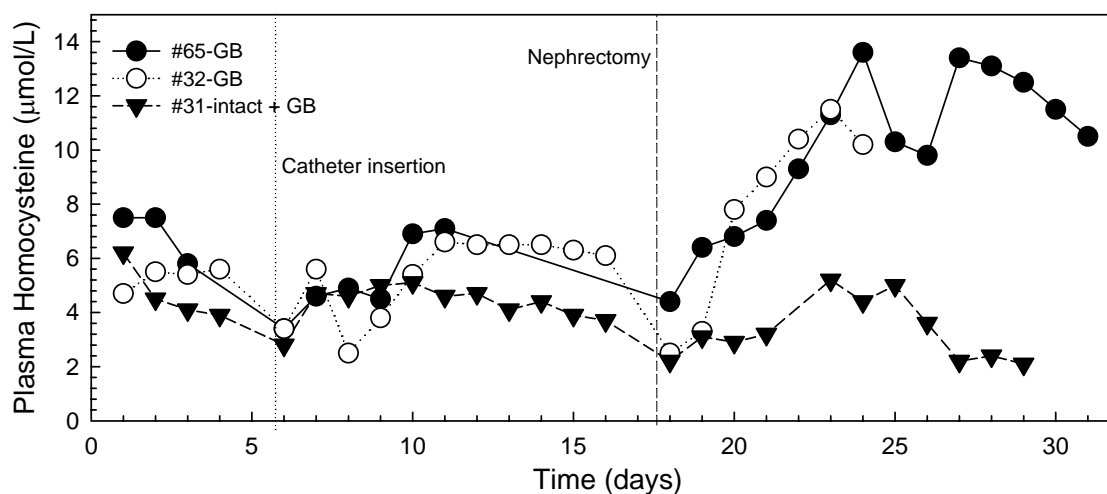


Figure 5.7: Daily plasma Hcy of sheep on GB treatment

5.4 DISCUSSION

5.4.1 CAPD sheep model

In this study a large-animal model of continuous ambulatory peritoneal dialysis (CAPD) was created. Different animal models, rabbits, rats and mice, have been used to investigate specific aspects of peritoneal dialysis (Schambye et al., 1992; Breborowicz et al., 1998; Zweers et al., 2001; Zheng et al., 2001; Hekking et al., 2003). However, experiments that have been performed using animal models for peritoneal dialysis, have lasted only a few hours with only one or two exchanges of dialysis fluid (Zweers et al., 2001; Hekking et al., 2003). Hekking et al. (2003) injected the dialysis fluid directly into the abdominal cavity rather than via an implanted catheter that is typical of human clinical subjects. To our knowledge this was the first attempt at generating a CAPD model in large animal (sheep).

Sheep were the animals of choice because they could be manipulated to more closely mimic the human clinical situation compared with other animal models (rats and rabbits); combining renal failure with several dialysis fluid exchanges per day, using fluid volumes and regimes similar to that of human dialysis patients. Animals were maintained on dialysis treatment for up to 14 days following the induction of acute renal failure by full bilateral nephrectomy. While this was achieved just in three animals, it was technically demanding and not without its difficulties. As in human patients, catheter patency, fluid loading and malnutrition were difficult to control.

Catheter blockage, a result of omental encasement, is not uncommon in animal model of peritoneal dialysis. Heparin coated catheters have been used in a chronic rat model of PD to prevent omental wrapping of the catheters (Zareie et al., 2004). Similarly, catheter patency was maintained in this study through a regime of daily flushing and loading of catheters with concentrated heparin and steroid solution. However, there are concerns about the use of heparin and steroid as they may interfere with the peritoneal membrane function (De Vriese et al., 2002).

Animals in this study, particularly 167 and 73, suffered from fluid loading that led to peripheral and pulmonary oedema, which manifested itself clinically as severe dyspnoea. This was presumably a result of high rate of water and electrolyte transport across the peritoneal membrane and loss of renal ultrafiltration. Although animal 167 survived the 14 day dialysis treatment period, it was clear from the post mortem results that the dialysis protocol needed to be modified for animals 75 and 73 in an effort to prevent the fluid retention from occurring. The modified regime reduced dwell times to 3 hours, with no fluid overnight. Nevertheless the new regime was only partially successful in preventing fluid loading and also control of plasma creatinine and urea concentrations was less successful than in animal 167 (Figure 5.4) suggesting inadequate dialysis.

An anephric haemodialysis sheep model studied by Eschbach et al., 1980, found that the model was less stable and harder to maintain and study than a partially nephrectomised model. A partially nephrectomised model however, has its disadvantages. Firstly, the level of renal failure and the time between sub-total nephrectomy and the requirement for dialysis treatment varies between individual animals. Secondly, it would also be difficult to maintain CAPD catheter patency for longer periods of time.

5.4.2 Dialysis with DMT containing fluid

It has been previously shown in rats that DMT, a synthetic glycine betaine analogue, is more effective at lowering plasma Hcy than an equivalent dose of GB (Slow et al., 2004) and as such had therapeutic potential as an Hcy lowering agent. This was evaluated in a CAPD sheep model with the addition of DMT to the dialysis fluid.

It was unexpected that sub-acute exposure to DMT would be toxic in sheep causing total consolidation and collapse of both lungs (Figure 5.6 (A)). There were no observed adverse side effects in rats following the subcutaneous administration of two doses of DMT (Slow et al., 2004). In a pilot study of CAPD sheep model, the effects of a single 10g subcutaneous dose of DMT in sheep was studied and no adverse side effects were observed (Slow, S., personal communication; unpublished). The reasons for the observed toxicity in the acute renal failure CAPD sheep model are not readily apparent, but could

be the result of several factors. Firstly, the toxicity observed in the sheep CAPD model might be a species-specific effect. In adult sheep, BHMT is present in the liver, pancreas and kidney, but the liver and pancreas are the only tissues that have been found to have significant BHMT activity, with the pancreas having the higher activity of the two tissues (Xue and Snoswell, 1986). In rats BHMT is found predominantly in the liver (McKeever et al., 1991) and activity is significantly higher than that of sheep liver BHMT (Xue and Snoswell, 1986). The different profile of tissue BHMT activity between rats and sheep may therefore be a significant factor in determining the metabolic fate of DMT. Interestingly, the expected de-methylated product of DMT, methylthioacetate, which was found in the plasma and urine of the DMT treated rats, was not observed in the plasma or dialysis fluid of the DMT treated sheep. This suggests that the DMT was not metabolised via BHMT in the sheep, and that the metabolic fate of DMT is different in sheep and rats. The presence of a rumen may also be a contributing factor, as the rumen microbes may metabolise DMT, potentially producing a toxic metabolite that may result in the lung pathology observed.

Secondly, the rats were only exposed to two doses of DMT and each dose was administered one-week apart (Slow et al., 2004). The rats received approximately 460 mg/kg of DMT per dose, while the sheep received approximately 500-800mg/kg of DMT per day, over the four dialysis exchanges (Table 5.1). Although the average time to death in the sheep was less than 48 hours, the sheep were continuously exposed to large doses of DMT for consecutive days. This combined with the possibility that DMT metabolism in sheep may be reduced, a result of a much lower liver BHMT activity, suggests that the sheep would have been exposed to high plasma DMT concentrations for longer than the rats. This is supported by the finding that the plasma DMT concentration in the rats 24 hours following administration was more than 20-fold lower (Slow et al., 2004) than that observed in the sheep after 48 hours (0.5-7.8 mmol/L; Table 5.1). The exposure to DMT, particularly high plasma concentrations, may have therefore been too short in the rats to detect any adverse effects. Finally, the route of administration may also be important (intra peritoneal in sheep; subcutaneous in rats). However, none of these factors explain

why sub-acute exposure to DMT via dialysis fluid affected only the lungs and not other organs.

5.4.3 Dialysis using GB containing fluid

Although oral supplementation with GB in humans has proven useful for lowering plasma Hcy concentrations (Wilcken et al., 1983; McGregor et al., 2002; Olthof et al., 2003), long-term compliance is problematic and diarrhoea has been reported during therapy (Knopman and Patterson, 2001). Consequently, we tried to explore the effectiveness of adding betaine to dialysis fluid as a means of increasing methyl donor availability for the treatment of hyperhomocysteinemia in CRF patients receiving dialysis.

Unlike DMT containing fluid, sub-acute exposure to GB via dialysis fluid had no adverse effects on any of the animals studied. It did not affect any of the organs, including lungs, contrary to that which was observed with DMT treatment. At post-mortem, the heart, liver, lungs and all other organs had a normal appearance and the lung tissue samples were similar to those treated with standard dialysis fluid (Figure 5.6 (B)). This proved that addition of GB to dialysis fluid does not cause any toxic effect in sheep. Even though there was catheter patency problems (Animal 32 and 31) and the dialysis had to be stopped, there were no indication that this was related to the addition of GB to the dialysis fluid.

Plasma Hcy concentration did not decrease with GB treatment in the nephrectomised animals, as expected. Though we could not definitively prove that addition of GB lowered homocysteine concentration, we were able to show that there was an increase in plasma GB concentration in animals, which proves that GB is effectively taken up from the dialysis fluid and enters the circulation. Animal 31 with normal kidney function showed a decrease in the plasma Hcy concentration with the GB treatment (Figure 5.7) and was less than half its pre-dialysis Hcy concentration by the time the dialysis was stopped.

The nephrectomised animals (32 & 65) had absolutely no renal function (acute renal failure), which may be a cause for sudden increase in Hcy concentration even though they were being treated with GB. The decrease in Hcy concentration observed in Animal 31, a situation similar to chronic renal failure patients who have residual renal function, suggests that addition of GB to dialysis fluid may be a useful option for treating hyperhomocysteinemia.

5.5 CONCLUSIONS

To our knowledge this was the first attempt at generating a large-animal CAPD model, and although it was only achieved in a small number of animals, it provides an important starting point for further development. A sheep CAPD model has several advantages over a small animal model as it allows researchers to study the same animal repeatedly in both control and uremic conditions. Although the acute renal failure CAPD sheep model proved difficult to maintain, the problems encountered are commonly experienced in human CAPD patients. Development of a chronic renal failure animal through partial nephrectomy or deliberate damage, rather than an acute renal failure model as described here could ameliorate these problems, but its feasibility also requires further investigation.

Dimethylthetin (DMT), a synthetic betaine analogue, was found to be toxic in sheep. Although the study here involved a small number of animals, it is clear that caution is required when using DMT. The safety of DMT as a therapeutic agent must be further assessed, preferably in multiple animal species, utilising varying doses and employing numerous administration routes.

Long-term oral supplementation with GB in chronic renal failure patients for lowering homocysteine concentration has been problematic and diarrhoea has been reported during therapy (Knopman and Patterson, 2001). Addition of GB in dialysis fluid for chronic renal failure patients can now be a viable option as we have shown that GB is not toxic to sheep when administered via dialysis fluid. This method may also help in lowering the

plasma Hcy concentration in CRF patients. Addition of GB to dialysis fluid for lowering plasma Hcy has never been tried before and our results can be a basis for further investigations.

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Chapter 6 - Effect of Compensatory Solutes on DNA Melting Temperatures and Their Use as PCR Enhancers

6.1 INTRODUCTION

6.1.1 DNA and PCR

Deoxyribonucleic acid (DNA), known as the molecule of heredity, contains the genetic information needed for the development of life. DNA, in its native state, exists as a double helix which is comprised of two single strands running antiparallel to each other and held together by hydrogen bonds (Figure 6.1). Each DNA strand comprises units called nucleotides, which in turn comprises a phosphate group, a sugar and a base. Four different nitrogenous bases are found in DNA, adenine (A), guanine (G), cytosine (C) and thymine (T), which are derivatives of two parent heterocyclic compounds, pyrimidine and purine. The successive nucleotides of each DNA strand are covalently linked to each other by a phosphodiester linkage while the two different strands are held together by hydrogen bonds that are formed between the complementary bases A with T (a double bond) and G with C (a triple bond).

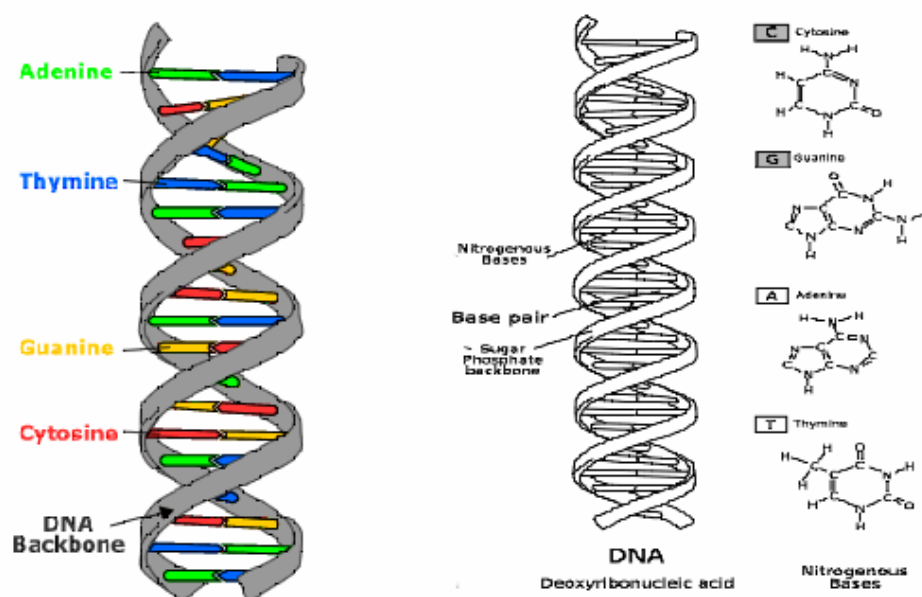


Figure 6.1: Double helix structure of a DNA and structures of nitrogenous bases (www.en.wikipedia.org and <http://evolution.berkeley.edu>)

Two sets of forces are responsible for holding the DNA double helix: (1) Hydrogen bonding between base pairs and (2) hydrophobic interactions, which shield the bases from water and cause them to be largely hidden within the double helix (Lehninger, 1982). The specific sequence of the four bases A, T, G and C are used for coding the genetic information. During replication of DNA *in vivo*, the DNA double helix is melted or dissociated and each separate DNA strand is used as a template for synthesizing a new strand. *In vitro*, the same process is mimicked by the polymerase chain reaction (PCR) (Newton and Graham, 1994).

PCR is a very popular biological technique which is used for detection of hereditary diseases, cloning of genes, forensic identification, etc. PCR allows the amplification of any specific DNA region. The amplification of a DNA template is achieved by using short, single stranded synthetic DNA molecules known as primers, which are complementary to the ends of the DNA fragment to be amplified.

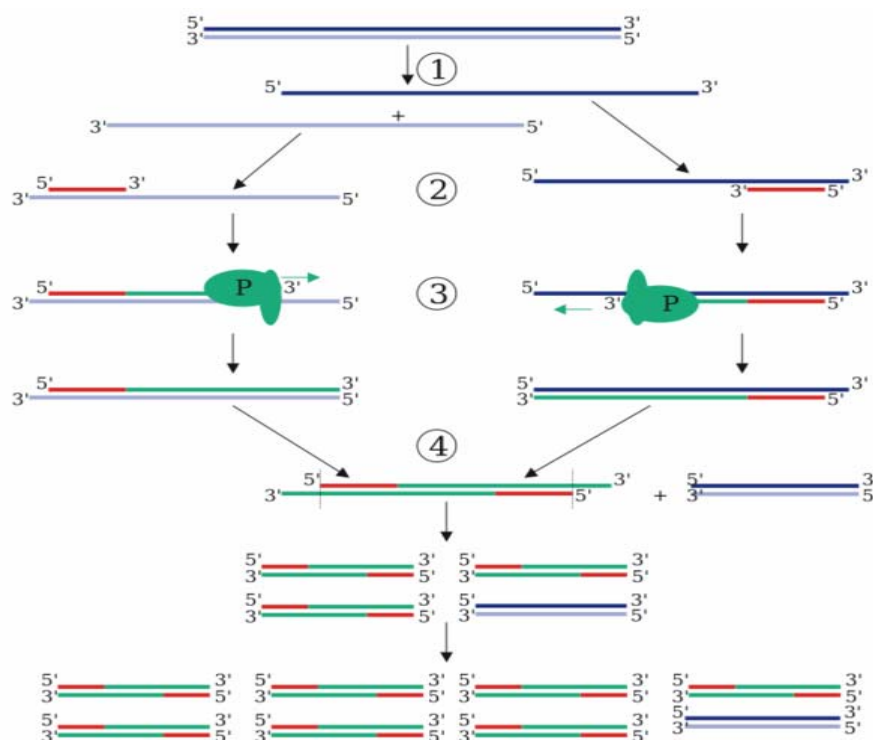


Figure 6.2: The polymerase chain reaction (PCR). (1) Denaturation of DNA double strands (2) Annealing of primers (3) Elongation by enzyme DNA polymerase 'P' (4) New DNA strands. (www.en.wikipedia.org).

During PCR, the double stranded DNA is separated into two single strands by heating, then temperature is lowered for the primers to anneal to the single stranded DNA templates and then an enzyme, DNA polymerase, extends the primers by incorporation of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. Thus, new DNA strands are synthesized that are complementary to the template strands. This kind of new strand synthesis can be repeated by heat denaturing the double-stranded DNA, annealing the primers by cooling and primer extension by DNA polymerase at a suitable temperature. Each repetition is called a PCR cycle and after 'n' cycles this results in 2^n -fold amplification of the DNA fragment. PCR is usually carried out in a thermal cycler, a machine that heats and cools the reaction tubes to the set temperatures.

6.1.2 DNA melting temperature (T_m)

High temperatures disrupt the hydrogen bonds between the base pairs and the hydrophobic interactions resulting in the denaturation of the DNA. The temperature at which the DNA double helix unwinds into two separate strands is known as the DNA melting temperature (T_m). Each species of DNA has a characteristic melting temperature. The GC base pair having three hydrogen bonds between them are more stable and require more heat energy to dissociate than the AT base pair with a double bond. Thus, the higher the content of GC base pairs in a DNA, the higher is its melting temperature (Newton and Graham, 1994).

DNA melting temperature plays a very important role in PCR amplifications. With GC-rich templates, the temperature for the denaturation step has to be very high and this sometimes results in reduced activity of DNA polymerase. In the past, scientists have used different solutes or destabilizers to reduce the DNA melting temperature. Tetraalkylammonium (TAA) compounds have been used as general destabilizers of DNA (von Hippel and Wong, 1965). Amino acids such as glycine and β -alanine have been observed to reduce the thermal stability of DNA macromolecule at concentrations of 0.0001-1.0 M (Aslanyan and Arutyunyan, 1984). Rees et al. (1993) showed that glycine betaine (GB) can eliminate the base pair composition dependence of DNA melting and thus reduce the melting temperatures. At molar concentrations, GB was shown to

destabilize the double helical conformation of DNA, and the melting temperature, T_m , decreases approximately linearly with increasing GB concentrations. A number of different polyols, including sorbitol, have also been shown to affect the thermal stability of calf thymus DNA by reducing its T_m (Vecchio et al., 1999). They showed that increasing the concentration of polyols decreased the denaturation enthalpy and denaturation temperature. Trehalose, a known compatible solute, has also been observed to reduce DNA melting temperatures substantially (Spiess et al., 2004).

The mechanism by which these solutes destabilize the DNA double helix is not properly understood and different mechanisms have been proposed for different group of solutes. TAA salts are strong electrolytes which are present in aqueous solutions as cations and anions, perturb the polyelectrolyte properties of the DNA resulting in denaturation (von Hippel and Wong, 1965). Amino acids at concentrations of 0.001-1.0 M are said to interact with DNA phosphate groups and destroy the spatial structure of water around DNA, bringing about the destabilization of macromolecule (Aslanyan and Arutyunyan, 1984). Osmolytes and compatible solutes such as GB and sorbitol, which are known to stabilize proteins under different environmental stresses, have been shown to destabilize DNA and reduce its melting temperature (Rees et al., 1993; Vecchio et al., 1999). To understand the isostabilizing effect of GB on DNA, von Hippel and co-workers (Rees et al., 1993) proposed that the effect was due to binding of the GB to the DNA major groove. This binding gives rise to weak and non-cooperative hydrophobic interactions with the methyl group of the thymines exposed in the major groove, which increases the local stability of AT base pairs and in turn reduces the stability difference between GC and AT base pairs. Thus, higher concentrations of GB reduce the melting temperature of a DNA by eliminating the base-pair composition dependence (Rees et al., 1993). Regarding the effect of polyols on DNA, it has been proposed that the hydroxyl groups of polyhydric alcohols compete with water for formation of hydrogen bonds with the side groups of the bases. This replacement of water by molecules of higher mass might amplify the thermal fluctuations of the polynucleotide resulting in destabilization of DNA double helix (Vecchio et al., 1999).

6.1.3 PCR Enhancers

PCR is one of the most important methods used in biological sciences. Though it is a very powerful and robust procedure, there are instances where a particular region of DNA proves difficult to amplify. The initial and critical step of the PCR, that is the melting of DNA double strands, depends mainly on the base pair composition of the template (Marmur and Doty, 1962). During amplification of targets rich in GC content high melting temperatures have to be used, which can be a limitation for the PCR and attempts to amplify GC rich targets can often result in little or no yield of the expected product (Frackman et al., 1998). To counter this, optimization of PCR by changing magnesium concentration, buffer pH, cycle number, and denaturing and annealing times have been tried. However, this has not been useful in all cases. Others have tried including a variety of additives and enhancing agents in the PCR mix in order to amplify GC rich templates and increase yields.

It has been shown that addition of glycine betaine in a PCR assay enhances/improves the amplification of GC-rich DNA sequences (Henke et al., 1997). Several other additives, for example DMSO (Frackman et al., 1998), low molecular weight amides (Chakrabarti and Schutt, 2001a) and low molecular weight sulfones (Chakrabarti and Schutt, 2001b) have also been used for enhancement of PCR amplification. Schnoor et al. (2004), investigated the effects of naturally occurring ectoine-type compatible solutes and their synthetic derivatives on DNA melting temperatures and PCR amplification of different templates. They showed that homoectoine, a synthetic derivative of L-ectoine, effectively enhanced PCR amplification of GC-rich DNA.

Detection of Fragile X syndrome by PCR analysis is an example of analysis of a GC-rich DNA template. Fragile X syndrome is a common cause of inherited mental retardation in humans and is caused by the expansion of the CGG trinucleotide repeats in the FMR1 (Fragile X mental retardation 1) gene. The FMR1 gene is located on the X chromosome and is responsible for making FMRP, a protein essential for normal brain functioning. In the normal population the FMR1 gene has between 6 and 50 CGG repeats. However, the

FMR1 mutations responsible for Fragile X syndrome result in approximately 50 to 200 CGG repeats (premutation) and full mutations result in more than 200 repeats (www.acmg.net). PCR analysis allows accurate determination of CGG repeat number for both normal alleles and mutant alleles of FMR1 gene. However, as this is a GC-rich DNA analysis, commercially available PCR enhancers (e.g. Roche GC-RICH PCR System) have to be used to obtain proper amplification of the DNA fragment. More information about Fragile X syndrome and its detection methods can be obtained in web pages cited in the references section of this chapter.

6.1.4 Objectives

The objectives of this project were to investigate the effects of novel synthetic compensatory solutes on DNA melting temperatures and their application as PCR enhancers. For DNA melting studies, we used three different DNA samples with varying GC content, *Clostridium Perfringens* (26.5% GC), Calf thymus (42% GC) and *Micrococcus lysodeikticus* (72% GC). After identifying the solutes which were effective in reducing the DNA melting, we tested their effectiveness as PCR enhancers in Fragile X PCR analysis and to check whether they would be able to replace a commercially available enhancer.

6.2 MATERIALS AND METHODS

Glycine betaine, DMSO, TMAO, trehalose, *Clostridium perfringens* DNA with 26.5% GC base pairs, calf-thymus DNA with 42% GC base pairs and *Micrococcus lysodeikticus* DNA with 72% GC base pairs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Synthetic compensatory solutes, PB, DB, HDB, HGB, CB-1, CB-2, CB-3, SB-2, SB-3, SB-4, CSB-1 and CSB-2 were synthesized as described in Chapter 3 of this thesis. *Taq* DNA polymerase (From *Thermus Aquaticus* BM, recombinant (*E. Coli*)) and GC-RICH PCR system kit were purchased from Roche Diagnostics GmbH, Germany. DNA marker ØX174 RF DNA/ Hae III fragments were purchased from Invitrogen. DNA templates for PCR analyses were gifted by Dr. Jeff Upton, Molecular Pathology, Canterbury Health Laboratories.

DNA stock solutions were prepared by dissolving the lyophilized DNA samples in 5 mM K_2HPO_4 buffer containing 0.1 mM Na_2EDTA (pH 7.5) and stored at 4°C for 48 hours.

6.2.1 DNA melting studies

All spectrophotometric studies were conducted at pH 7.5 in solutions containing 5 mM K_2HPO_4 and 0.1 mM Na_2EDTA . DNA melting profiles were monitored at 260 nm on a UV spectrophotometer (Cary 4000, Varian, Melbourne, Australia) equipped with a Peltier temperature controller and using the ‘Thermal’ software (Varian). Before monitoring the melting profiles, the equipment was blanked with buffer or the particular compensatory solution. For all three DNA samples, 100 μL DNA solution (final concentration 50 $\mu\text{g}/\text{mL}$) was mixed with 900 μL buffer/ compensatory solution (final concentration 2 M) and the melting profile was monitored starting from 35°C. The temperature was raised at 1°C/min and data was collected at every 0.1°C rise in temperature. The DNA melting temperatures (T_m) were determined by the ‘Thermal’ software using derivative calculations.

6.2.2 Effect of compensatory solutes on *Taq* DNA polymerase

To determine the effect of compensatory solutes on the activity of *Taq* DNA polymerase, 10 μL of enzyme solutions (5x diluted) were mixed with 10 μL of different compensatory solutions (2M final) and incubated at room temperature for 60 minutes. Aliquots (5 μL) of these mixtures were then used as enzyme solution for PCR analyses. To determine whether compensatory solutions were able to stabilize *Taq* DNA polymerase against heat, 10 μL of enzyme solutions (5x diluted) were mixed with 10 μL of different compensatory solutions (2M final) and incubated at 95°C for 120 minutes in a Perkin–Elmer thermo cycler. Aliquots (5 μL) of these mixtures were then used as enzyme solution for PCR analyses. For control experiments, 10 μL of enzyme solutions were mixed with 10 μL of Roche buffer (10x diluted). The PCR reaction mixture contained 10x diluted Roche buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , pH 8.3), 0.5 μM CTE2F forward primer, 0.5 μM HP2R reverse primer, 10 ng/ μL gDNA template, 0.2 mM each dNTP and 0.025 U/ μL *Taq* polymerase. dNTP’s were purchased from Roche and primers from Invitrogen. The sequences of the primers were as follows, forward 5’-

GGGAGCGCCACCCCTAACATGCTATT-3' and reverse 5'-CTCTCCCAGGCAGACTGGCC-3'. Amplification reaction was carried out on a Perkin-Elmer thermocycler using 50 μ L solutions in 200 μ L thin-walled tubes. Amplifications were run under the following conditions: Initial denaturation at 94°C for 2 min and annealing at 60°C for 30 sec. This was followed by 30 cycles of denaturation at 94°C for 30 sec and anneal/extension at 65°C for 1 min. Finally the mixture was maintained at 65°C for 10 min and cooled down to 20°C. Amplification products were analyzed by agarose gel electrophoresis on 2% agarose gels in which 7 μ L of reaction products were loaded with 3 μ L of loading buffer. Gels were stained with ethidium bromide, visualized on a UV transilluminator (Gel Doc 2000, Bio-Rad), and documented by photography.

6.2.3 Fragile X PCR analysis

Fragile X PCR analyses were carried out to determine the effectiveness of synthetic compensatory solutes for their application as PCR enhancers. Fragile X PCR analysis is a GC-rich system and usually a GC-rich resolution solution (abbreviated as “RS” further in this thesis) supplied by GC-Rich PCR System kit, Roche Diagnostics, Germany, is used as an enhancer to obtain proper amplification of CGG repeats in the FMR1 gene.

The PCR reaction mix was as follows: GC-rich reaction buffer (5x diluted containing 1.5 mM MgCl₂ and DMSO), 1.5 M GC-rich resolution solution (RS), 0.25 μ M FMR1 forward primer, 0.25 μ M FAM-FMR1 reverse primer, 0.2 mM each dNTP, 0.04 U/ μ L Platinum *Taq* High Fidelity™ polymerase (Invitrogen) 10 ng/ μ L DNA template. dNTP's were purchased from Roche and primers from Invitrogen. The sequences of the primers were as follows, forward 5'-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3' and reverse 5'-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3'. Amplification reaction was carried out on an Eppendorf MasterCycler thermocycler using 50 μ L solutions in 200 μ L thin-walled tubes. Amplifications were run under the following conditions: Initial denaturation 98°C for 5 min and annealing at 60°C for 1 min, followed by 3 cycles of extension at 72°C for 1 min, denaturation at 98°C for 30 sec and annealing at 60°C for 30 sec. Then the amplification was run for 33 cycles with denaturation at 96°C for 15 sec, annealing at 67°C for 1 min and extension at 72°C for 1 min. Finally the mixture was

held at 72°C for 10 min and then cooled down to 20°C. Amplification products were analyzed by agarose gel electrophoresis on 3% agarose gels in which 7 µL of reaction products were loaded with 3 µL of loading buffer. Gels were stained with ethidium bromide, visualized on a UV transilluminator and documented by photography.

Seven different Fragile X DNA templates with varying number of CGG repeats were available at the Molecular Pathology Department of Canterbury Health Laboratories. Firstly, Fragile X PCR analysis was done using all the seven templates to determine the exact amplification products to be expected.

Secondly, to determine whether synthetic compensatory solutions could replace GC-rich resolution solution (Roche), different concentrations of synthetic compensatory solutions (prepared in reaction buffer) were added in the Fragile X PCR reaction mixture. For control experiments, different concentrations of the resolution solution were added to the reaction mixture.

Thirdly, the effect of mixing compensatory solutions with the resolution solution was investigated. For this, 5 M compensatory solutions were mixed with 5 M resolution solution (Roche) in different volume fractions and added to the reaction mix. Also, different concentrations of compensatory solutions were prepared in the resolution solution instead of buffer, and then added to the reaction mix.

Lastly, as magnesium is a co-factor required for the activity of thermostable DNA polymerase and the concentration of magnesium is an important factor that can affect the success of the amplification (www.promega.com), different concentrations of MgCl₂ were added along with the compensatory solutions to determine if better Fragile X PCR products could be obtained.

6.3 RESULTS

6.3.1 DNA melting studies

Table 6.1 lists the DNA melting temperatures, T_m , of three different DNA samples (with varying GC content) in the presence of different 2 M compensatory solutions. Trehalose was used at 1 M final concentration because of solubility constraints. Also listed are the approximate changes in T_m (°C) compared to the T_m of a DNA sample in the absence of any additive. In the absence of any additive the T_m of *Clostridium perfringens* (CP) DNA was approximately 58°C, calf thymus (CT) DNA was approximately 65°C and *Micrococcus lysodeikticus* (ML) was approximately 80°C. It is evident from the results that as the GC% of DNA increases, its T_m increases.

As expected, GB, dimethyl sulfoxide (DMSO), sorbitol and trehalose were able to destabilize the different DNA samples. However, their destabilization effect on the DNA samples was different. The destabilization effect of GB seemed to increase with the increasing GC content of the DNA samples. A similar effect was observed in the presence of DMSO. Trehalose showed an opposite effect with its effectiveness to reduce T_m of the DNA decreasing with increasing GC content. The effect of sorbitol did not change though the GC content of the DNA increased. In the presence of sorbitol, the change in T_m remained approximately the same (Table 6.1). Trimethyl-*N*-oxide (TMAO) had very little effect on all three DNA samples.

Interesting results were obtained when the DNA samples were heated in the presence of synthetic compensatory solutes. Propio betaine increased the T_m of both CP and CT DNA, and had a negligible effect on ML DNA. The stabilizing effect of propio betaine seemed to decrease with the increase in GC content of the DNA samples. A similar stabilization effect was observed in the presence of hydroxyl group containing solutes, DB, HDB and HGB. HGB was the best stabilizer of the three, and it increased the T_m of all the DNA samples significantly, though its stabilizing effect seemed to decrease with increasing GC content.

Table 6.1: $T_m \pm$ SEM of different DNA samples in the presence of 2M compensatory solutions. Changes in T_m ($^{\circ}\text{C}$) listed in the table are the approximate changes in melting temperature compared to T_m of that particular DNA in the absence of any additive (no addition)

Additive (2 M)	<i>Clostridium perfringens</i> (26.5% GC)		Calf thymus (42.0% GC)		<i>Micrococcus lysodeikticus</i> (72.0% GC)	
	T_m , $^{\circ}\text{C}$	Change in T_m , $^{\circ}\text{C}$	T_m , $^{\circ}\text{C}$	Change in T_m , $^{\circ}\text{C}$	T_m , $^{\circ}\text{C}$	Change in T_m , $^{\circ}\text{C}$
No addition	57.9 ± 1.0	0.0	64.8 ± 0.7	0.0	80.1 ± 0.1	0.0
GB	54.6 ± 0.9	-3.3	60.2 ± 0.3	-4.5	72.4 ± 0.2	-7.7
DMSO	54.2 ± 0.4	-3.7	59.9 ± 0.7	-4.9	74.6 ± 0.5	-5.5
TMAO	60.1 ± 1.3	2.1	66.3 ± 0.3	1.5	79.6 ± 1.0	-0.5
Sorbitol	52.3 ± 0.3	-5.6	58.0 ± 0.6	-6.8	73.8 ± 1.1	-6.3
Trehalose*	53.4 ± 0.4	-4.5	59.7 ± 0.4	-5.1	77.0 ± 0.6	-3.1
PB	64.8 ± 1.5	6.9	68.2 ± 3.4	3.4	79.2 ± 1.0	-0.9
DB	67.6 ± 0.8	9.7	72.4 ± 1.2	7.7	82.3 ± 0.7	2.2
HDB	68.6 ± 0.4	10.7	69.8 ± 0.2	5.0	81.6 ± 0.8	1.5
HGB	73.2 ± 1.0	15.3	77.6 ± 1.1	12.8	84.8 ± 0.2	4.7
CB-1	56.2 ± 0.8	-1.7	59.7 ± 0.5	-5.1	70.3 ± 0.3	-9.8
<i>Sulfobetaines</i>						
SB-2	51.6 ± 1.1	-6.3	56.5 ± 0.7	-8.3	67.0 ± 1.0	-13.1
SB-3	69.3 ± 1.5	11.4	71.5 ± 0.5	6.8	81.9 ± 0.7	1.8
SB-4	52.6 ± 3.4	-5.4	59.2 ± 2.0	-5.6	71.1 ± 1.9	-9.0
CSB-1	54.3 ± 0.4	-3.6	59.7 ± 0.3	-5.1	70.5 ± 0.5	-9.6
CSB-2	52.8 ± 1.8	-5.1	56.1 ± 0.9	-8.6	64.8 ± 0.7	-15.3

* Trehalose was used at 1 M concentration

Sulfobetaines were generally effective in reducing the T_m of the DNA's, except for SB-3. In the presence of SB-2 and CSB-2, maximum reduction in T_m of all the three DNA samples was observed. Their destabilization effect increased sharply with the increase in the GC content and they reduced the T_m of the DNA samples almost twice as much as GB and sorbitol (Table 6.1). SB-4 and CSB-1 were also better in reducing the T_m compared to the natural solutes, but they were not as effective as SB-2 and CSB-2. Surprisingly, SB-3 increased the T_m of the DNA samples instead of reducing their T_m , as expected. The stabilization effect of SB-3 was similar to that of HDB.

From the melting profiles of CT DNA (Figure 6.3), we observe that in the absence of any additive the melting curve is broad. In the presence of GB, DMSO and sorbitol, which reduced the T_m , the melting curves are broad and similar to that of DNA without an additive. However, in the presence of synthetic solutes, both stabilizers and destabilizers, the melting curves seem to sharpen and are less broad. This is evident from the melting curves of SB-2, CSB-1 and CSB-2, which are very effective in reducing the T_m of the DNA samples. Similarly, melting curves in the presence of solutes that increased the T_m (stabilizers), such as propio betaine, DB and HDB, were narrower.

The relation between change in melting temperatures (T_m) and GC content of DNA samples in the presence of various solutes, both destabilizers and stabilizers, is shown in Figure 6.4. From the figure we observe that there is a particular trend in the way in which these solutes affect DNA stability with the increase in GC %. The change in T_m decreases with the increase in GC%, irrespective of whether the solute is stabilizing or destabilizing the DNA. Solute such as HDB and HGB stabilize CP DNA (26.5 % GC) effectively and increase its T_m by approximately 14°C. However, as the GC content increases (e.g. ML DNA with 72% GC), their stabilization effect becomes minimal. On the other hand, the destabilization effect by solutes SB-2 and CSB-2 increases with the GC content and they reduce the T_m further. Thus, we observe that the effect of stabilization decreases and effect of destabilization increases with the increase in DNA GC content.

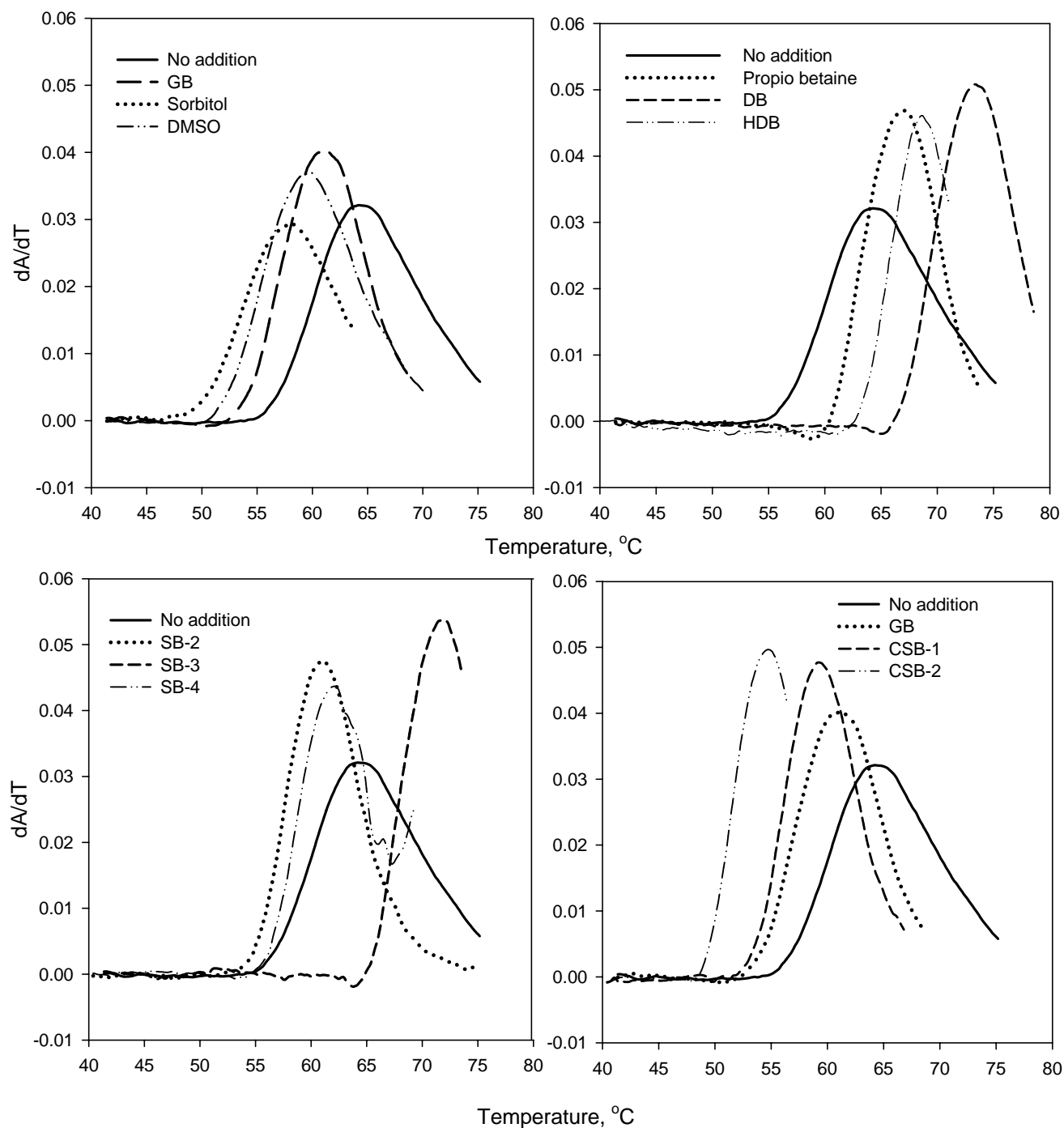


Figure 6.3: Melting profiles (derivative curves) of calf thymus DNA (42% GC) in the presence of different 2 M compensatory solutions. Melting was monitored by UV absorbance at 260 nm using Cary 4000 (Varian) UV spectrophotometer.

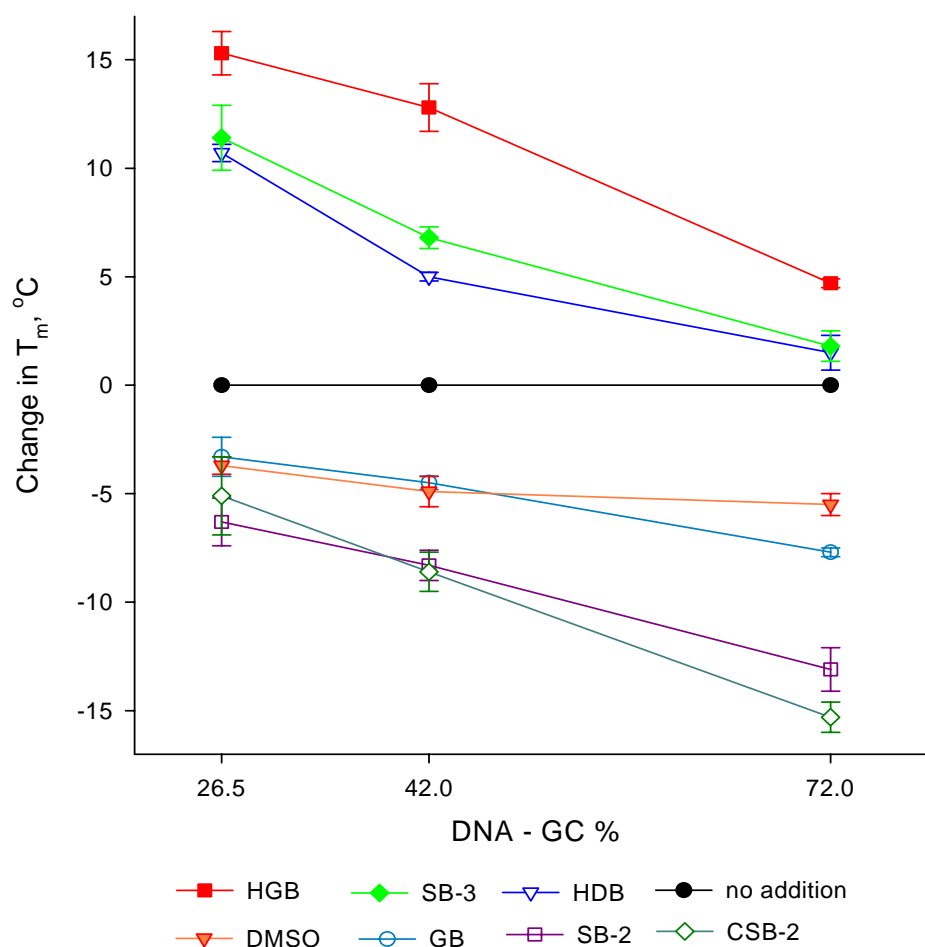


Figure 6.4: Relation between change in T_m ($^{\circ}\text{C}$) and GC% of DNA samples in the presence of different 2 M compensatory solutes.

6.3.2 Effect of compensatory solutes on *Taq* DNA polymerase

The effect of compensatory solutes on the activity of *Taq* DNA polymerase is shown in Figure 6.5. *Taq* polymerase was incubated at room temperature for 60 min in the presence of 2 M compensatory solutes and then an aliquot of this enzyme solution was used in the PCR analysis. Except for CB-2 and CB-3, none of the solutes inhibit the activity of the enzyme. Amplification products similar to that of control are obtained even though the enzyme was incubated in presence of 2 M solutes. Initially, no products were found in the presence of HGB, CB-2, CB-3 and SB-1 (Gel 1, Figure 6.5). To reconfirm

these results, the experiments were repeated a few times and subsequently it was consistently found that HGB and SB-1 do not inhibit the activity of *Taq* polymerase (Gel 3, Figure 6.5).

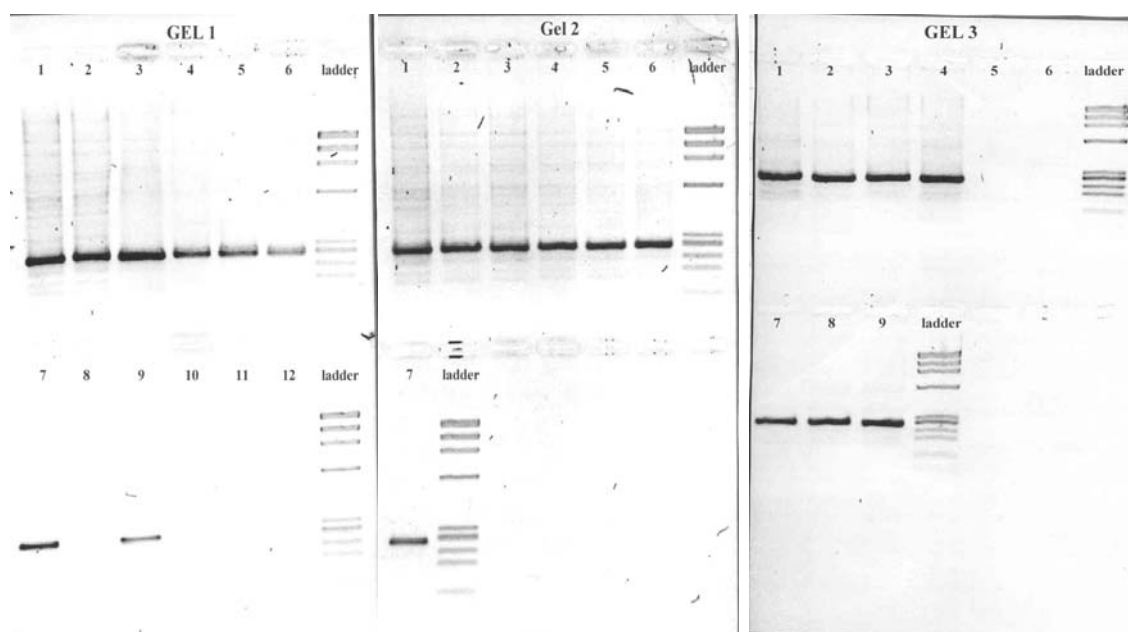


Figure 6.5: Effect of compensatory solutes on normal activity of *Taq* DNA polymerase.

Gel 1: (1) Control/expected product (2) GB (3) Sorbitol (4) Trehalose (5) Propio betaine (6) DB (7) HDB (8) HGB (9) CB-1 (10) CB-2 (11) CB-3 (12) SB-1 **Gel 2:** (1) Control/expected product (2) SB-2 (3) SB-3 (4) SB-4 (5) CSB-1 (6) CSB-2 (7) DMT **Gel 3:** (1) Control (2) HDB (3) HGB (4) CB-1 (5) CB-2 (6) CB-3 (7) SB-1 (8) SB-4 (9) CSB-2. (Ladder: ØX174)

Figure 6.6 shows the ability of compensatory solutes to stabilize *Taq* polymerase against heat. Enzyme solutions were incubated with 2 M compensatory solutions at 95°C for 2 hours and then used in PCR analysis. Though the majority of the compensatory solutes do not affect the normal activity of the enzyme, only a few were able to protect the enzyme against extreme heat. In the absence of any solutes (lane 2, Gel 4 and 5) *Taq* polymerase is completely inactivated by heat and no amplification products are found. However, in the presence of 2 M GB, sorbitol, trehalose, SB-1, SB-2, SB-3, CSB-1 and DMT, amplification products similar to that of untreated enzyme are found. These results

show that a few solutes are effective in protecting *Taq* polymerase against extreme heat. Solutes such as propiobetaine, HGB, HDB and CB-1, which were very good stabilizers of LDH and chymotrypsin against heat were unable to protect *Taq* polymerase from inactivation by prolonged exposure to heat.

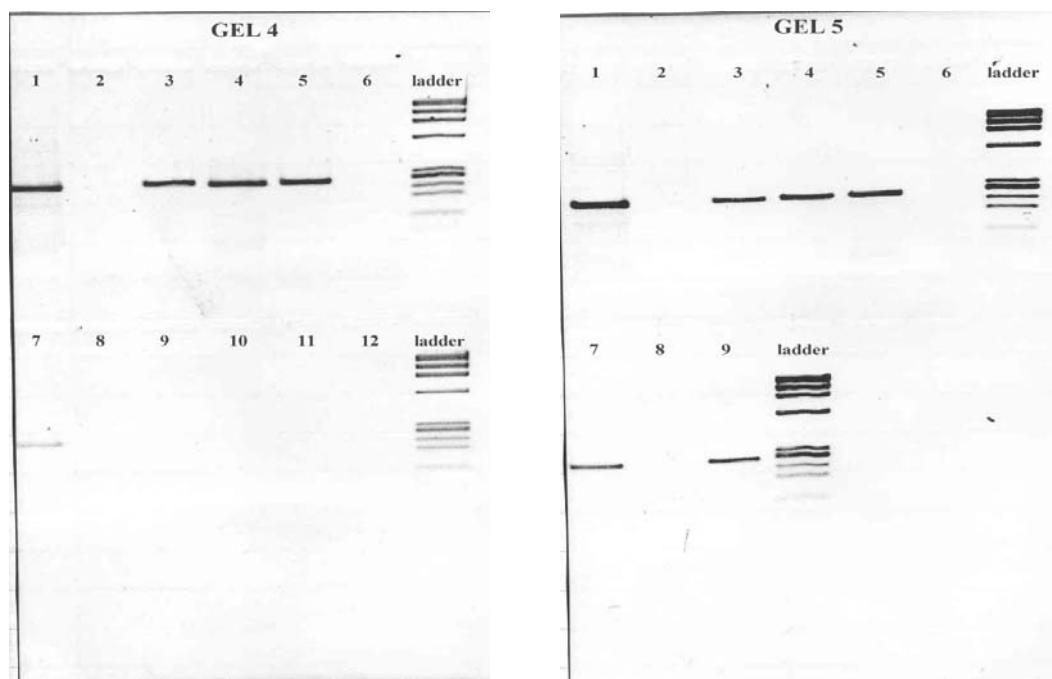


Figure 6.6: Stabilization effect of compensatory solutes against heat treatment of *Taq* polymerase. The enzyme was heated at 95°C for 2 hours in the presence of 2 M solutes and then used in the PCR. **Gel 4:** (1) Untreated control/expected product (2) Heat treated control (3) GB (4) Sorbitol (5) Trehalose (6) Propio betaine (7) DB (8) HDB (9) HGB (10) CB-1 (11) CB-2 (12) CB-3 **Gel 5:** (1) Untreated control/expected product (2) Heat treated control (3) SB-1 (4) SB-2 (5) SB-3 (6) SB-4 (7) CSB-1 (8) CSB-2 (9) DMT. (Ladder: ØX174)

6.3.3 Fragile X PCR analysis: Compensatory solutes as PCR enhancers

Fragile X PCR analyses of the seven different DNA samples (available at Molecular Pathology, Canterbury Health Laboratories) were done using Roche supplied GC-Rich Resolution Solution as the PCR enhancer, and the expected products are shown in Figure 6.7. The sizes of PCR products show the approximate number of CGG repeats present in each allele of the individual being tested. The PCR products in lane 1, 2, 4, 5 and 7 on

Gel 6 (Figure 6.7) show premutation of the FMR1 gene and approximate number of CGG repeats. Full mutations ($n > 200$) are not detected by PCR analysis and this is shown on lane 3 and 6, where only normal allele has been amplified.

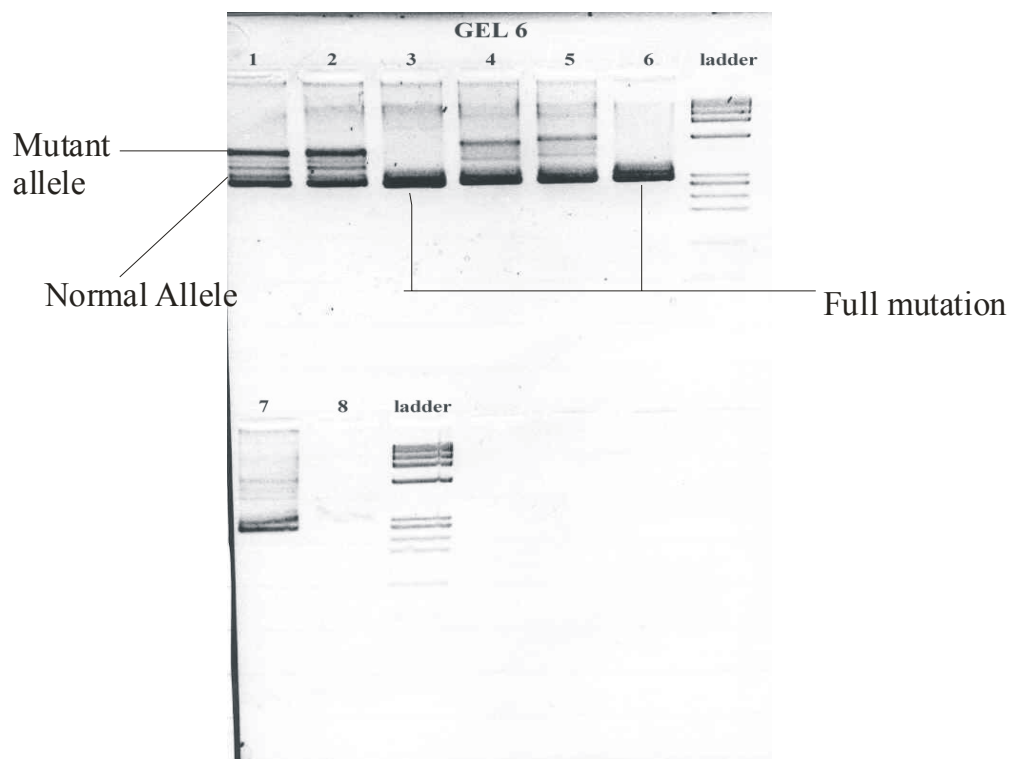


Figure 6.7: Expected amplification products of Fragile X PCR analysis using different DNA templates. **Gel 6:** (1) DNA #1 (2) DNA #2 (3) DNA #3 (4) DNA #4 (5) DNA #5 (6) DNA #6 (7) DNA #7 (8) No DNA (control). (*Note: These analyses were carried out using Roche supplied GC-Rich Resolution Solution as the PCR enhancer*) (Ladder: ØX174)

In DNA samples in which premutations are detected (lane 1, 2, 4, 5 and 7), we can see two bands of PCR products. The lower band represents the normal allele and the upper band shows the results from the mutant allele. DNA # 1 has a premutation with approximately 55-65 CGG repeats (shown by upper band on lane 1). Similarly, DNA # 2, 4, 5 and 7 have premutations with approximately 60-70, 80-100, 90-110, and 100-200 CGG repeats respectively. As the amplification of these CGG repeats on mutant alleles is difficult in a fragile X PCR analysis, enhancers like GC-Rich Resolution Solution are always added in the reaction mixture.

We chose synthetic solutes SB-2 and CSB-1, and the natural solute GB, to study the effects of replacing GC-Rich Resolution Solution in a standard fragile X PCR. In the standard method, GC-Rich Resolution Solution is used at a final concentration of 1.5 M. However, as we were not sure about the exact concentration of our synthetic solutes that would give optimal results, we tried adding our solutions in a range of concentrations to determine their effect. Figure 6.8 and 6.9 show the results of these experiments.

For control experiment, GC-rich resolution solution was diluted to a range of concentrations starting at 1.5 M and the results are shown in Gel 7. We observe that the expected PCR products along with the CGG repeats are only obtained with the addition of 1.5 M resolution solution.

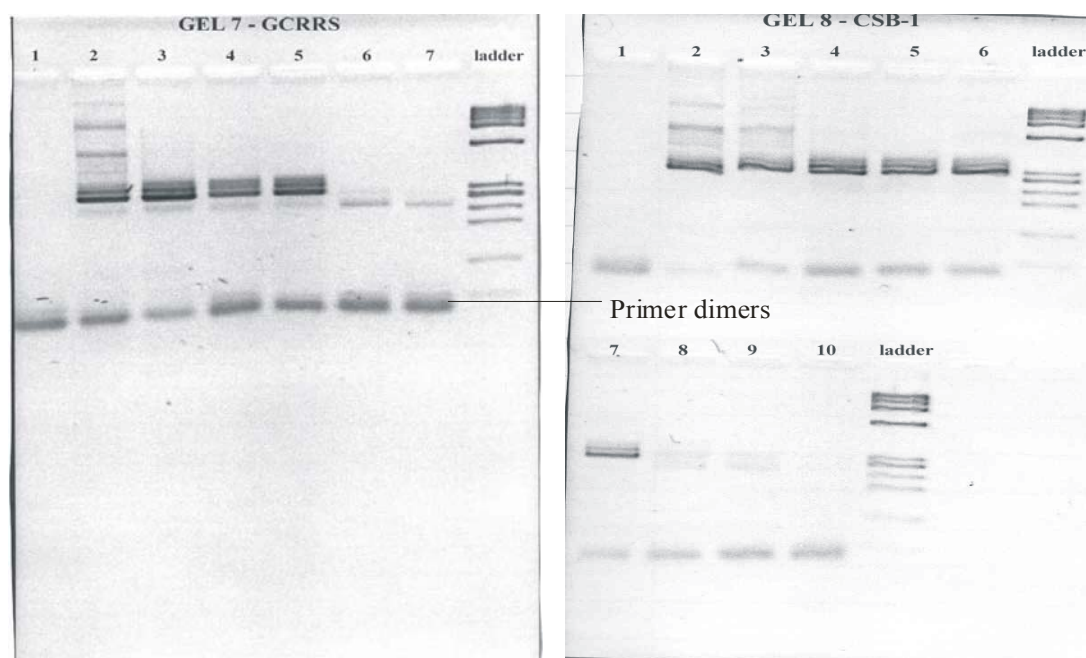


Figure 6.8: Synthetic compensatory solutes as Fragile X PCR enhancers, replacing GC-rich resolution solution (GCRRS or RS). Gel 7- varying concentrations of RS. Gel 8 – varying concentrations of CSB-1. **GEL 7:** (1) No enhancer (2) 1.5 M RS/expected product (3) 1.25 M RS (4) 1 M RS (5) 0.75 M RS (6) 0.5 M RS (7) 0.25 M RS **GEL 8:** (1) No enhancer (2) 1.5 M RS/expected product (3) 2 M CSB-1 (4) 1.75 M CSB-1 (5) 1.5 M CSB-1 (6) 1.25 M CSB-1 (7) 1 M CSB-1 (8) 0.75 M CSB-1 (9) 0.5 M CSB-1 (10) 0.25 M CSB-1 (*Note: DNA # 5 was used for both the gels, Ladder: ØX174*)

As the concentration of resolution solution decreases, we observe that the PCR fails to amplify the expanded CGG repeats required to detect Fragile X syndrome, and at concentrations less than 0.5 M, amplification of normal allele also fails.

Gel 8 (Figure 6.8) shows the effect of replacing resolution solution with the synthetic solute CSB-1. CSB-1 was used in a concentration range starting from 2 M (final). Results show that the addition of 2 M CSB-1 (lane 3) in the reaction mixture gives the expected products along with the amplification of expanded CGG repeats and the products are similar to those obtained with the addition of resolution solution (lane 2). However, this amplification is concentration dependent and as the concentration of CSB-1 decreases there is an abrupt termination of the ability to amplify CGG repeats in the premutation range.

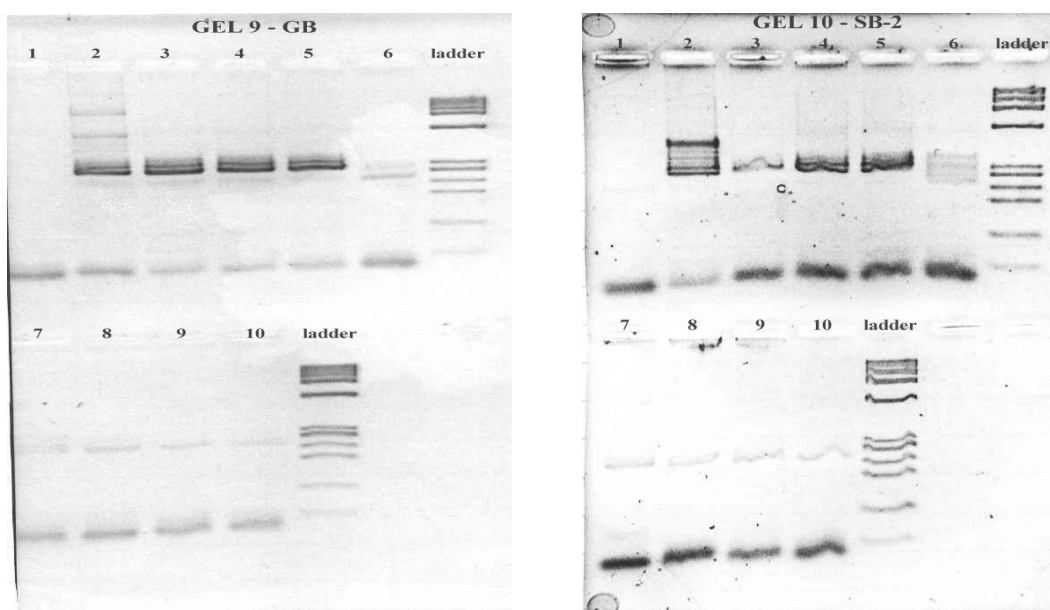


Figure 6.9: Synthetic compensatory solutes as fragile X PCR enhancers, replacing GC-rich resolution solution (GCRRS or RS). Gel 9- varying concentrations of GB and Gel 10 – varying concentrations of SB-2. **Gel 9:** (1) No enhancer (2) 1.5 M RS/expected product (3) 2 M GB (4) 1.75 M GB (5) 1.5 M GB (6) 1.25 M GB (7) 1 M GB (8) 0.75 M GB (9) 0.5 M GB (10) 0.25 M GB **Gel 10:** (1) No enhancer (2) 1.5 M RS/expected product (3) 2 M SB-2 (4) 1.75 M SB-2 (5) 1.5 M SB-2 (6) 1.25 M SB-2 (7) 1 M SB-2 (8) 0.75 M SB-2 (9) 0.5 M SB-2 (10) 0.25 M SB-2 (Note: DNA # 5 was used for Gel 9 and DNA # 1 was used for Gel 10, Ladder: ØX174)

Addition of GB (Gel 9, Figure 6.9) instead of resolution solution did not result in the amplification of the expanded CGG repeats, though the normal allele is effectively amplified. Due to the limited quantity of DNA samples available for the experiments, a different DNA sample, DNA # 1, was used for experiments with SB-2.

Results of addition of SB-2 to the PCR mix are shown in Gel 10 (Figure 6.9). SB-2 fails to produce the expected amplification products at 2 M concentration. Good amplification products are observed when SB-2 is added at concentrations of 1.75 M and 1.5 M (lane 4 and 5, Gel 10, Figure 6.9). Though there is the amplification of the normal allele, SB-2 fails to amplify the CGG repeats needed to detect the premutation in the gene. However, the appearance of light coloured bands in lane 4 and 5 indicate that there may be minimal amplification of the CGG repeats. No amplification products are detected after a further decrease in the concentration of SB-2.

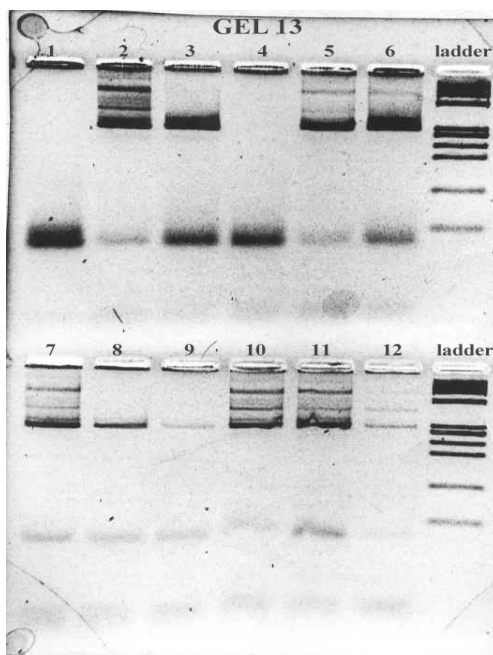


Figure 6.10: Effect of mixing 1.5 M resolution solution and 1.5 M SB-2 and CSB-1 solutions. **GEL 13:** (1) No enhancer (2) RS/expected product (3) 7.5 µL RS + 7.5 µL SB-2 (4) 5 µL RS + 10 µL SB-2 (5) 7.5 µL RS + 7.5 µL CSB-1 (6) 5 µL RS + 10 µL CSB-1 (7) 0.5M SB-2 (8) 1M SB-2 (9) 2M SB-2 (10) 0.5M CSB-1 (11) 1M CSB-1 (12) 2M CSB-1 (Note: Solutions of compensatory solutes in column 7-12 were prepared in resolution solution instead of buffer, Ladder: ØX174)

Resolution solution and compensatory solutions (equimolar) were mixed in different volume fractions and their effect on the fragile X PCR was studied (lane 3-6, figure 6.10). None of the mixtures were able to produce the expected amplification products. Mixing equal volumes of CSB-1 and resolution solution gave satisfactory products, but the products were not similar to those products with just the resolution solution (lane 2). We also tried adding solutions of compensatory solutes prepared in the resolution solution, instead of buffer. Results of these attempts are shown in lane 7-12, Gel 13, Figure 6.10. As we observe from the figure, even these attempts did not yield products similar to the resolution solution (lane 2).

Figure 6.11 shows the results of adding different concentrations of MgCl_2 along with 2 M CSB-1 and 1.5 M SB-2. No significant result could be obtained with these experiments when DNA # 5 was used (Gel 14, Figure 6.11). However, when DNA # 1 was used (Gel 16, Figure 6.11), we observe that adding different concentrations of MgCl_2 along with 2 M CSB-1 gives products similar to that of resolution solution. With the addition of 1.5 mM and 1 mM MgCl_2 with 2 M CSB-1 (lane 4 and 5, Gel 16), we see that amplification products are better than those obtained with the addition of resolution solution (lane 2, Gel 16). CGG repeats that help in detection of mutation in the FMR1 gene were also very well amplified when 2 M CSB-1 and 1.5 mM MgCl_2 were used in combination during the Fragile X PCR analysis.

These results show that synthetic compensatory solutes CSB-1 and SB-2 have the potential to replace the more expensive GC-rich resolution solution (Roche) as a PCR enhancer, especially for analysis of DNA templates that are rich in GC content. However, due to time constraint of this project, the experiments were not continued further. More experiments and PCR optimization are required for use of these solutes effectively.

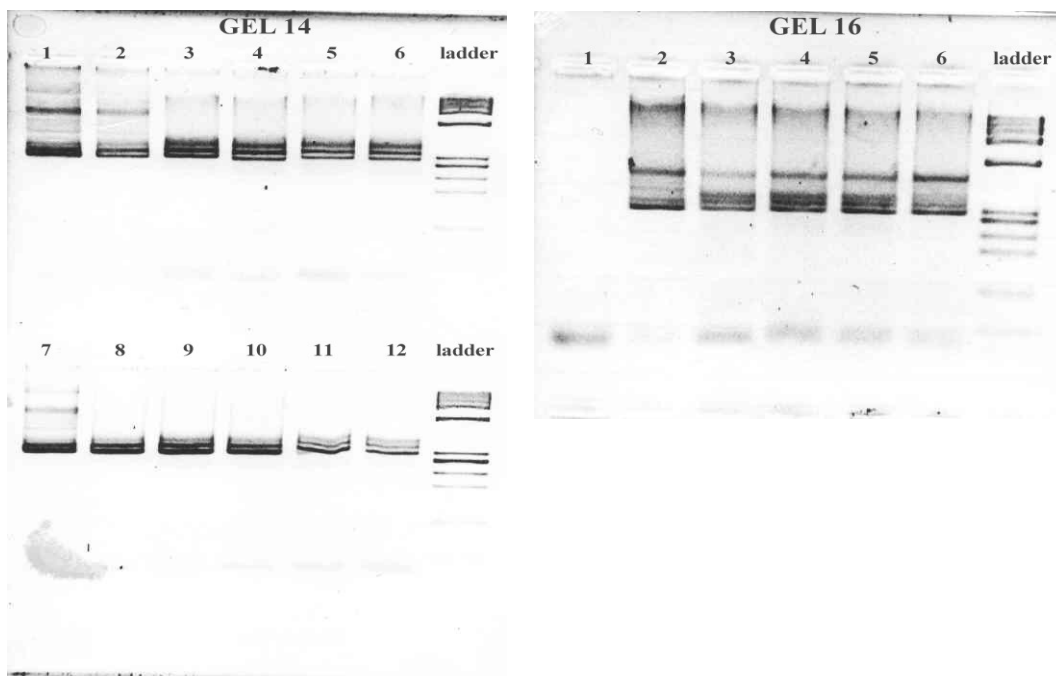


Figure 6.11: Effect of addition different concentrations of MgCl_2 along with 2 M CSB-1 and 1.5 M SB-2 to fragile X PCR analysis. **GEL 14:** (1) RS/expected product (2) CSB-1 (3) CSB-1 + 0.5 mM MgCl_2 (4) CSB-1 + 1 mM MgCl_2 (5) CSB-1 + 1.5 mM MgCl_2 (6) CSB-1 + 2 mM MgCl_2 (7) RS (8) SB-2 (9) SB-2 + 0.5 mM MgCl_2 (10) SB-2 + 1 mM MgCl_2 (11) SB-2 + 1.5 mM MgCl_2 (12) SB-2 + 2 mM MgCl_2 **GEL 16:** (1) No enhancer (2) RS/expected product (3) CSB-1 + 2 M mM MgCl_2 (4) CSB-1 + 1.5 mM MgCl_2 (5) CSB-1 + 1 mM MgCl_2 (6) CSB-1 + 0.5 mM MgCl_2 (Note: DNA # 5 was used for Gel 14 and DNA # 1 for Gel 16)

6.4 DISCUSSION

Destabilization of DNA or reducing the T_m is very important for methods such as PCR where the initial step is the separation (melting) of double stranded DNA into single strands by heat denaturation. Researchers in the past have tried using different additives like tetraalkylammonium salts (von Hippel and Wong, 1965), glycine betaine (Rees et al., 1993), proline (Rajendrakumar et al., 1997), polyols (Vecchio et al., 1999) and trehalose (Spiess et al., 2004). In this chapter the results of effects of different compensatory solutes, both natural and synthetic, on three different DNA samples with varying GC

content are presented. Some of these synthetic solutes destabilize the DNA better than most of the known natural solutes and some solutes stabilize the DNA very well against heat.

There seems to be no general mechanism by which these additives destabilize the DNA and reduce the T_m . Different mechanisms have been proposed to explain the destabilization effect of different additives. Except for homoectoine (Schnoor et al., 2004), most of the other studies have used naturally occurring osmolytes and compatible solutes for destabilizing the DNA. These osmolytes and compatible solutes have been extensively studied for their ability to stabilize proteins and biological assemblies without affecting their normal cellular functions. It is interesting that the same solutes are being used to destabilize a macromolecule like DNA.

von Hippel and co-workers (1993) proposed that GB binds to the major groove of the DNA which causes the destabilization of the DNA double helix. Hong et al. (2004) studying the preferential interactions of GB and urea with DNA, deduced that urea is a effective DNA denaturant because of its favorable interactions with the polar amide surfaces of G, C, and T bases exposed in denaturation, while GB is a specific GC denaturant because of its favorable interaction with the G or C surface, or both. This argument of interaction of GB with GC bases is supported by the observation that the destabilizing effect of GB on DNA increases with increasing fraction of GC base pairs (Rees et al., 1993). Even our results of melting of DNA in the presence of 2 M GB supports these results and shows that the destabilizing effect of GB increases with the increase in DNA GC% (Table 6.1).

Results from Chapter 4 showed that propio betaine, DB, HDB and HGB are very good protein stabilizers, and results in this chapter show that they stabilize and protect the DNA as well. On the other hand, we observe that compensatory solutes GB, sorbitol and trehalose stabilize enzymes against heat (Chapter 4), but here we observe that they destabilize the DNA and reduce its melting temperature. Another interesting result is that

propio betaine which only differs from GB in its structure with an extra CH_2 between the charges stabilizes DNA, whereas GB destabilizes the DNA.

Vecchio et al. (1999), in their study on effects of polyols on thermal stability of calf thymus DNA proposed that the presence of hydroxyl groups makes polyols capable of destabilizing the DNA. This was due to the hydroxyl groups forming hydrogen bonds with the DNA bases, both in the minor and major grooves, increasing the dehydration of DNA. They concluded that polyols which have more hydroxyl groups have a higher destabilizing effect. However, our results show a completely opposite trend. In our results we see that with the increase in the number of hydroxyl groups in the compensatory solutes increases its DNA stabilizing effect instead of destabilization. HGB, which has two hydroxyl groups, stabilizes the DNA samples more than HDB and DB, which have one hydroxyl group each (Table 6.1). This stabilization effect by compensatory solutes with hydroxyl groups support the results of Schnoor et al. (2004) who showed that β -hydroxyectoine, a compatible solute with hydroxyl group, increased the melting temperatures of double stranded DNA. Schnoor et al. (2004) also suggested that their results indicate that the carboxylic group or the zwitterionic structure is important for lowering the melting temperature. However, that is not the case with our results where propio betaine, DB, HDB and HGB tend to increase the melting temperature though they are zwitterionic and have carboxylic group in their structure.

Sulfobetaines considered in our study were very effective in destabilizing the DNA samples, except for SB-3. Due to non-availability of the solute, SB-1 was not used for DNA melting studies. CSB-1 and CSB-2 (cyclic sulfobetaines) which were synthesized in the later stages of this project were included for DNA melting studies. Our results show that sulfobetaines SB-2, SB-4, CSB-1 and CSB-2 are very effective in destabilizing the DNA and they reduce the T_m twice as much as GB and sorbitol. The structural similarity between SB-2, SB-4, CSB-1 and CSB-2 is that they all have a C_3 bridge between S and N. However, we are unable to explain why SB-3 stabilizes the DNA instead of destabilization. The only structural difference between SB-3 and SB-2 is an extra CH_2 (C_4 bridge) between the charges, but still they show a completely opposite

effect (see Appendix A for structures of solutes). Another important observation from our results is that sulfobetaines SB-2 and SB-4 do not seem to stabilize the DNA similar to HDB and HGB even though they have hydroxyl groups in their structure. SB-4 with two hydroxyl groups in its structure destabilizes the DNA, whereas HGB, also with two hydroxyl groups, stabilizes the DNA. In addition, the difference between these two synthetic solutes is that SB-4 is a sulfonic acid analogue and HGB is a carboxylic acid analogue.

These results show that the effect of compensatory solutes on DNA and their effect on proteins are completely different mechanisms. The influence of these solutes on nucleic acids is different compared to proteins. More systematic studies with DNA are needed to understand the exact mode of action of these solutes.

Based on the effect of our synthetic compensatory solutes on the melting temperatures of different double stranded DNAs, we studied their effectiveness as PCR enhancers in fragile X PCR, a GC-rich PCR analysis. Glycine betaine (Henke et al., 1997) and DMSO (Frackman et al., 1998) have been used to improve the amplification of GC-rich DNA sequences. A recent study by Spiess et al. (2004) showed that the compatible solute trehalose greatly facilitates the PCR of GC-rich templates by reducing the DNA melting temperatures and thermostabilizing the *Taq* polymerase. All these studies have chosen their PCR enhancing additives based on their effectiveness to lower the DNA melting temperature.

The results of DNA melting studies identified a number of synthetic solutes that are twice as effective as GB, DMSO and trehalose in reducing the melting temperatures. Before using them as enhancers in a GC-rich PCR, it was decided to determine their effect on the activity of *Taq* polymerase. For this reason the enzyme was incubated at room temperature in the presence of synthetic solutes and then used the enzyme in a standard PCR. Most of the solutes did not alter the activity of *Taq* polymerase, except for CB-2 and CB-3. As described in Chapter 3, CB-2 and CB-3 form acidic solutions and that this

change in pH would have inhibited the enzyme's activity or by changing the pH of the PCR reaction mixture, which is very critical for a PCR.

Taq DNA polymerase is an enzyme originally isolated from the thermophilic eubacterium *Thermus aquaticus* BM. As it is derived from a thermophilic organism, *Taq* polymerase can withstand very high temperatures of a PCR. However in a GC-rich PCR, the denaturation temperature is very high and the enzyme has to endure high temperatures for short intervals during the approximate 2 hours of a PCR. There is a possibility that *Taq* polymerase can lose activity when exposed to such high temperatures, which in turn would affect the yield of amplification products. In Chapter 4 of this thesis it was shown that some of our synthetic solutes are very good enzyme stabilizers against heat. Thus, it was decided to study the stabilizing effect of synthetic compensatory solutes on *Taq* polymerase against heat stress. *Taq* polymerase solution was incubated with 2 M compensatory solutions at 95°C for two hours and then used in a PCR. Results showed that only a few solutes were able to protect *Taq* polymerase against heat and helped it retain its activity. Solute such as propio betaine, HDB, HGB and CB-1, which were very good stabilizers of LDH and chymotrypsin against heat, failed to protect *Taq* polymerase. This failure to protect may be due to the lack of stability of the solutes themselves against very high temperatures (see Chapter 7 for stability of solutes). Compensatory solutes had to withstand 95°C for two hours during incubation and also further endure the high temperatures of the PCR; these high temperatures might have caused the synthetic solutes to decompose and form their starting materials, which would have inhibited the enzyme or affected the PCR reaction mixture.

From the results of the above experiments we were able to identify two synthetic solutes, SB-2 and CSB-1, which could act as PCR enhancers and decided to use them in a Fragile X PCR. In a standard Fragile X PCR, Roche supplied GC-Rich Resolution Solution is used as a PCR enhancer to obtain the amplification of the (CGG)_n repeats. The GC-rich resolution solution is usually supplied in a GC-Rich System kit from Roche and is quite expensive to purchase. So, we decided to replace the resolution solution with SB-2 and CSB-1 solutions in the Fragile X PCR and determine if we could get similar

amplification products as the resolution solution. We also included GB in this study due to its known application as a PCR enhancer.

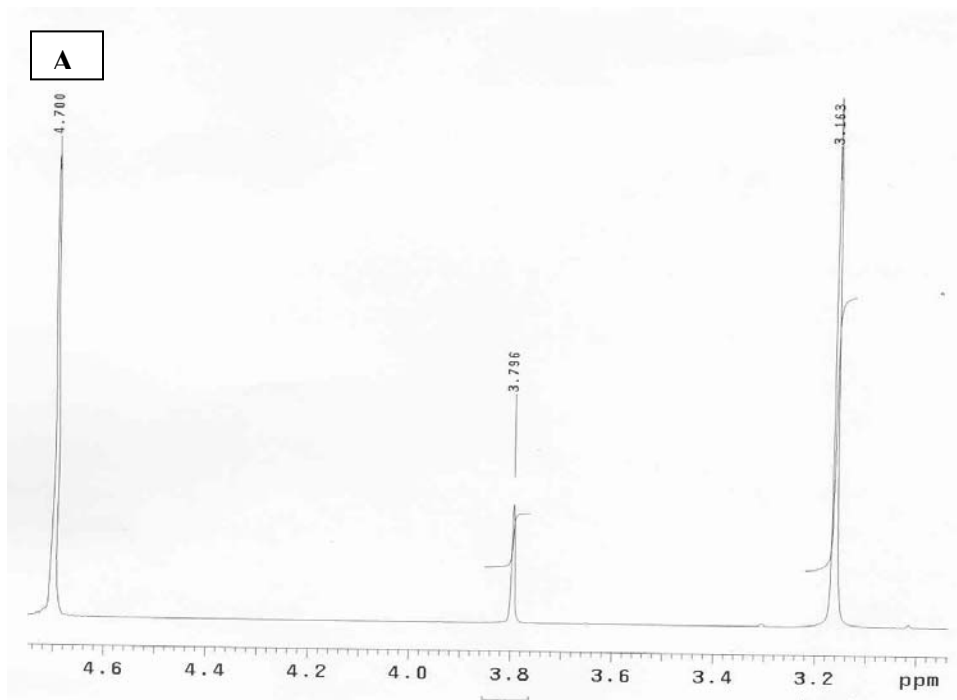
Our results have shown that 2 M CSB-1 gives the same kind of amplification products as that of 1.5 M Roche resolution solution. SB-1 and GB solutions did not produce similar products as they failed to amplify the CGG repeats of the mutant allele. However, SB-1 seems have the potential to be a PCR enhancer as there were indications in the results that CGG repeats were being amplified.

The GC-rich resolution solution is supplied by Roche, and the company does not reveal the ingredients of the solution. To investigate the identity of this commercial additive, we did a NMR analysis of the resolution solution (5 M) and compared it with the NMR of glycine betaine (5 M). The positions of the peaks in both the NMR spectra (Figure 6.12) indicate that the GB solution and resolution solution are same in their chemical composition. Even the sizes of the peak suggest that the two solutions are present in approximately the same concentration.

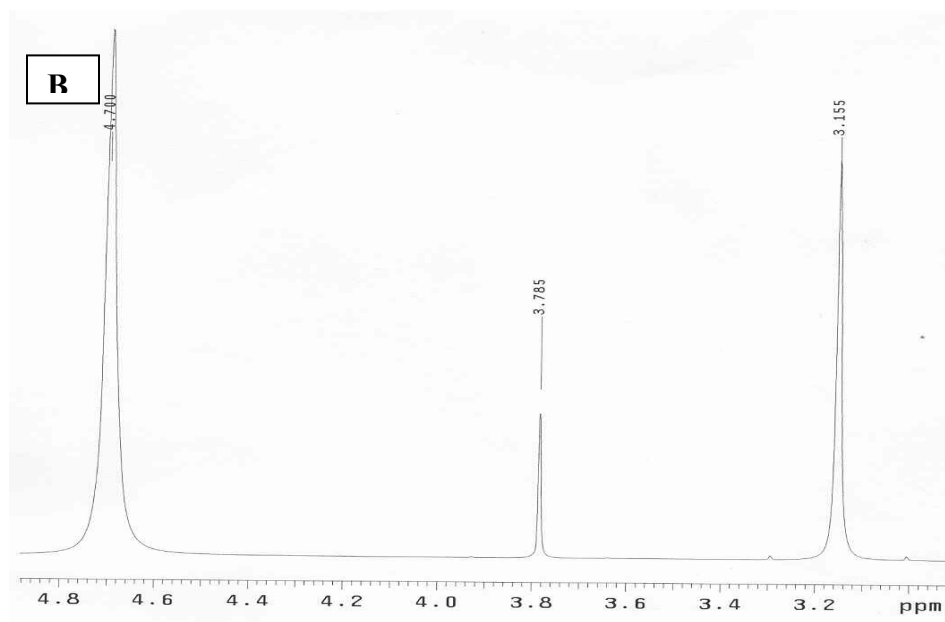
In NMR spectra we are only able to identify the organic protons. We decided to get the resolution solution analyzed for other additives. We sent the a sample of Resolution Solution to Canterbury Health Laboratories to check if there were traces of Na, K, Cl, Phos, Mg, and Mn. Results came out to be negative for all the elements tested. These analyses demonstrated that the Roche supplied GC-rich resolution solution is GB solution. However, though the Resolution Solution is presumably GB, we could not determine why a GB solution that was prepared in our laboratory did not have a similar enhancing effect on the Fragile X PCR.

Our attempts to get better Fragile X PCR products by mixing synthetic compensatory solutions, SB-2 and CSB-1, with resolution solution did not yield any promising results. As magnesium concentration is very important for a PCR, we also tried adding different concentrations of MgCl_2 along with our compensatory solutions. Results showed that mixing 2 M CSB-1 with 1 mM MgCl_2 results in exactly similar amplification products as

that of resolution solution. However, there was difference in results when two different DNA templates were used, which we are unable to explain.



NMR spectra (GC-Rich Resolution solution): ^1H NMR (D_2O , 500 MHz) δ 3.15 (9 H, s, $(\text{CH}_3)_3$), 3.78 (2 H, s, CH_2)



NMR spectra (Glycine betaine): ^1H NMR (D_2O , 500 MHz) δ 3.16 (9 H, s, $(\text{CH}_3)_3$), 3.79 (2 H, s, CH_2)

Figure 6.12: NMR spectra (A) Roche supplied 5 M GC-Rich Resolution solution (B) 5 M Glycine betaine solution. *Note: The peak at 4.7 ppm is water.*

Though PCR is a very robust procedure, it needs proper optimization. There are a number of variables such as magnesium concentration, buffer pH, enzyme concentration, template quality and quantity and thermal cycling parameters that need to be changed for every PCR. In our study, we chose a pre-optimized PCR, Fragile X, and tried to replace the commercial enhancer solution with our compensatory solutions. Our results have shown that SB-2 and CSB-1 reduce the DNA melting temperatures more than GB (main ingredient of resolution solution). However, we did not change the denaturation and annealing temperatures during thermal cycling, which might be the reason for not getting the exact amplification products as that of the resolution solution.

Results of this chapter have identified three synthetic solutes, SB-2, CSB-1 and CSB-2, which have the potential to replace commercial enhancers like the GC-rich resolution solution. We have studied the PCR enhancing effect of these solutes in only one GC-rich PCR system. It is suggested that more PCR optimization, and also studying the effects of these solutes in a different GC-rich PCR analysis would help in proving them as effective PCR enhancers.

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Chapter 7 - Physical Properties of Compensatory Solutes and Theoretical Calculations of Their Interactions with Water

7.1 INTRODUCTION

In the previous chapters, the effect of different compensatory solutes on proteins and DNA melting temperatures, and applications of compensatory solutes as PCR enhancers and therapeutic agents, was discussed. However, most of the synthetic solutes that have been detailed in this thesis are completely new and very little physical data is available for them. In this chapter results of density and viscosity measurements, apparent hydration numbers and the stability of the solutes themselves are presented. Theoretical calculations of interactions of water with different compensatory solutes by Dr. Robert Maclagan, Chemistry Department, University of Canterbury, New Zealand, are also included.

It was observed (Chapter 4) that compensatory solutes have different effects on different proteins and sometimes the protective effect can be protein or stress specific. Several theories have been proposed to explain the mechanism by which osmolytes or compatible solutes stabilize proteins. One of the most widely accepted mechanisms is the preferential exclusion mechanism proposed by Timasheff and co-workers (2.2.1, Chapter 2). One of the common adaptation strategies developed by living systems against hostile environments is to modify the properties of the solvent, usually water, in such a way as to exclude undesirable solutes from solution (Galinski, 1993). Stabilizing solutes (co-solvents) accumulated at very high concentrations are known to modify the water in the cell (Timasheff, 1993). Depending on solute/co-solvent's effect on the structure of water they are classified as 'Kosmotropes' (order maker) and 'Chaotropes' (Order breaker).

Kosmotropic co-solvents (e.g. glycine betaine) are more polar than water and they enhance the structure of water due to their ability to form hydrogen bonds (Galinski et al., 1997). For this reason they interact with water molecules rather than with non-polar protein side-chains, which leads to an effective preferential exclusion from the solvation

shell of proteins (Timasheff and Arakawa, 1997). Chaotropic co-solvents (e.g. urea) are less polar than water and their presence in solution leads to an energetically unfavorable disruption of water structure. Thus chaotropic co-solvents are excluded from bulk water and they preferentially bind to the protein side chains (Timasheff and Arakawa, 1997; Timasheff, 2002). In a theory proposed by Wiggins (1997), it has been suggested that water, in the presence of compensatory solutes and proteins, displays different properties. According to this theory two types of water structure can be distinguished: dense water in the hydration sphere of proteins and less dense or bulk water, which is preferred by the compensatory solutes.

It is evident from the proposed mechanisms of protein stabilization that the interaction of solutes/co-solvents with water plays a very important role in understanding the mechanism of protection. As the majority of the compensatory solutes described in this thesis are new, we decided to calculate the apparent hydration number, H , for different solutes and determine whether hydration number plays any role in the effect of solute. Hydration number, H , is usually used to express the amount of hydration of a solute, and is defined as the number of water molecules present in a hydration envelope of one molecule of solute (Vand, 1947). *The hydration envelope can be regarded as consisting of water molecules held to the molecule of the solute by certain binding forces, as a result of which an activation energy E is to be overcome by the molecules leaving the envelope* (Vand, 1947). The method of estimation of apparent hydration numbers for various aqueous solutions has been described by Edward (1957). Hydration numbers have also been used to study aqueous saccharide solutions (Kiyosawa, 1988).

Maclagan et al. (2004) carried out several theoretical calculations to understand the interaction of compensatory solutes with water. These calculations illustrated the variety of computational chemistry methods that can be applied to understand biological systems. Some of the calculations that they carried out were:

(1) Calculations of gas-phase structures that gave information about the charge distribution, dipole moments of the molecules and polarizability; and

(2) Calculations of the interaction energy of the solute molecules with a water molecule, which when compared with water-water interaction energy, showed whether the H-bonds in water will be broken by the solute molecule.

Results of these calculations suggested that compensatory solute molecules, while uncharged, often have a large dipole moment, which could lead to the modification of water structure close to the solute molecule (Maclagan et al., 2004). As we were interested to know more about the interaction of water with our novel synthetic compensatory solutes, we asked Dr. Robert Maclagan to do similar calculations with a few of our synthetic solutes.

It has been observed that some of the synthetic solutes that were good stabilizers of LDH and chymotrypsin failed to protect *Taq* polymerase against extreme heat stress (Chapter 6). Due to the suspicion that some of the synthetic solutes are not stable enough to withstand very high temperatures, it was decided to study the stability of the solutes using an HPLC system, which will be described further in this chapter. Measurement of density and viscosity of compensatory solutes will also be described.

7.2 MATERIALS AND METHODS

7.2.1 Stability of compensatory solutes

Stability of the compensatory solutes was determined using an HPLC system (Figure 7.1). Glass test tubes with screw caps were filled with 5 mL of 100 mM compensatory solutions and were incubated at 90 °C for 2 hours in a temperature block. During incubation, 100 µL aliquots of the solutions were taken every 30 minutes and mixed with 300 µl acetonitrile in HPLC vials and then transferred to the auto-sampler.

The experimental set-up contained one HPLC pump, Shimadzu LC-10 ADVP. The mobile phase, acetonitrile water mixture (3:1), passed through a membrane degassing module to the pump (1.0 mL/min), and then to an auto sampler, Shimadzu SIL10AXL fitted with a 400µL loop.

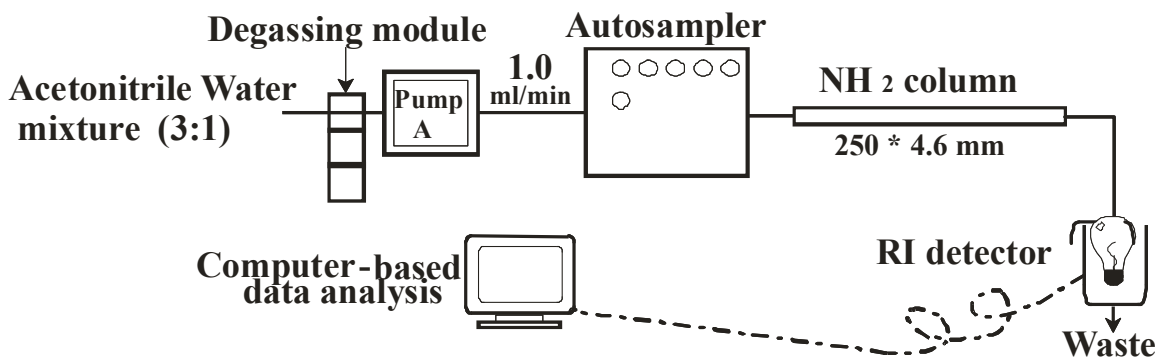


Figure 7.1: Experimental set-up

The auto-sampler, controlled by a system controller (Shimadzu SCL-10A) injected 40 μ L sample into the stream. The sample then passed through a HPLC column (Shodex Asahipak NH₂P-50), and the outlet from the column was measured using a RI detector (Jasco). The detector output was monitored using DELTA Chromatography System (Digital solutions, Brisbane, Australia). Area under the peak was also measured using the DELTA Chromatography system. Due to inconsistencies in results, RI detector was replaced by a UV detector (UVIS 200, Linear systems). Carboxylic acid analogues of betaine were then analyzed by UV detection at 249 nm using naphthacyl triflate for derivatization and a Merck Aluspher Alumina column, as described by Storer et al., 2006.

7.2.2 Density measurements

The density of synthetic compensatory solutions was measured using an Anton Parr Density meter using 23% w/w (kg solute/kg solution) compensatory solutions. The Anton Parr Density meter is a vibrating U-tube density meter, which works by counting the oscillations of a vibrating U-tube. According to Hooke's law, the period of spring is directly proportional to the square root of the mass. Likewise, with a constant volume U-tube, the mass or density is linearly related to the square of the period of oscillation. The density meter gives a read out of the period of oscillation for the U-tube, by which the value of density of the sample can be found.

The water bath heater was first turned on and set at a constant temperature of 22°C. The cell was cleaned thoroughly and dried by connecting the plastic tube from the pump to

the top syringe connection. The pump was then turned off and the period for air recorded (τ_a). A syringe was filled with distilled water, connected to the bottom connection of the U-tube and approximately 1 mL was injected. The period for water was then recorded (τ_w). The water in the tube was removed and remaining water flushed out using the air pump. Sample solutions were incubated for five minutes at 22°C in the water bath and then injected into the U-tube using a syringe. The period for the sample was recorded (τ_s). Two readings for the each sample were recorded to minimize the error. The density of the sample (ρ_s) was obtained from:

$$\rho_s = \rho_w + \left(\frac{\rho_w - \rho_a}{\tau_w^2 - \tau_a^2} \right) (\tau_s^2 - \tau_w^2) \quad (7.1)$$

Where ρ_w and ρ_a are the densities of water and air at 22 °C.

7.2.3 Viscosity measurements

Viscosity of all the compensatory solutions was measured using a Cannon-Manning Semi-Micro Viscometer (Size 100, Cannon Instrument Co., USA). 23% w/w solutions were used for measuring the viscosity and all measurements were done at a constant temperature of 22°C. Kinematic viscosity in mm²/s of each sample was calculated by multiplying the efflux time by the viscometer constant (0.015 mm²/s²)

7.2.4 Calculation of apparent hydration number (H) of compensatory solutes

Apparent hydration number (H) of each solute was calculated using their density and viscosity. It is easy to express the amount of hydration of each solute in solution as an average number ' H ' of water molecules present in the hydration envelope of one molecule of the solute. All solute particles are assumed spherical for these calculations. The following steps were followed to calculate the apparent hydration number, H .

Step 1:

Mass fraction (kg solute/kg solution) was calculated using:

$$\text{Mass fraction, } \omega = \frac{0.3 \times \frac{\text{Mol. wt. (unhydrated)}}{\text{Mol. wt. (hydrated)}}}{1 + 0.3 \times \frac{\text{Mol. wt. (unhydrated)}}{\text{Mol. wt. (hydrated)}}} \quad (7.2)$$

Step 2:

From mass fraction, density of solute, ρ_{solute} , was found using:

$$\rho_{\text{solute}} (\text{kg} / \text{m}^3) = \frac{\omega}{\left(\frac{1}{\rho_s} - \frac{1 - \omega}{\rho_w} \right)} \quad (7.3)$$

Where ρ_s and ρ_w are the densities of solution and water respectively.

Step 3:

Molar volume of the solute from density, V_{density} (m^3/mol), was calculated using:

$$V_{\text{density}} = \frac{\text{Mol. wt. (unhydrated)}}{\rho_{\text{solute}}} \quad (7.4)$$

Step 4:

Dynamic viscosity (Pa.s) = Kinematic viscosity x ρ_s

Step 5:

Relative viscosity, $\eta_{\text{rel}} = \frac{\text{Dynamic viscosity}}{\text{Viscosity of water at } 22^\circ \text{C} (\eta_w)}$

Step 6:

Assuming all solute particles to be spherical, we can use Einstein's equation

$$\eta_{\text{rel}} = \frac{(1 + 0.5\phi)}{(1 - \phi)^2} \quad (7.5)$$

Where ϕ is the total volume fraction ($\text{m}^3 \text{ solute} / \text{m}^3 \text{ solution}$). Substituting for η_{rel} from Step 5, value of ϕ was solved.

Step 7:

Molar volume of solute from viscosity, $V_{\text{viscosity}}$ (m^3/mol), was calculated using:

$$V_{\text{viscosity}} = \frac{\phi \times \text{Mol.wt.}(\text{unhydrated})}{\rho_{\text{solution}} \times \omega} \quad (7.6)$$

Step 8:

Molar volume of water, V_{water} (m^3/mol), was obtained from

$$V_{\text{water}} = \frac{0.01802}{\rho_w}$$

Where ρ_w is the density of water at 22°C.

Step 9:

The apparent hydration number, H , or the No. of water molecules per solute molecule, was calculated using:

$$H = \frac{V_{\text{viscosity}} - V_{\text{density}}}{V_{\text{water}}}$$

7.2.5 Theoretical Calculations

HDB, HGB, CB-1 and SB-2 were chosen for determination of gas-phase structures. The calculations were performed using the GAUSSIAN03 program (Frisch et al., 2003). The structures were determined at HF/6-31G, MP2/6-31G, and B3LYP/6-31G levels of theory. The charge on each atom was calculated from the calculated wavefunction using a procedure first suggested by Mulliken (Mulliken, 1955) and the charges are termed Mulliken populations. Dipole moments and polarizability were calculated from the electronic wavefunction.

7.3 RESULTS

7.3.1 Stability of compensatory solutes

Table 7.1 lists the rate of decomposition of different compensatory solutes per minute when 100 mM solutions were incubated at 90°C for two hours in a temperature block. Aliquots of these solutions were collected every 30 minutes during the incubation and analyzed in the HPLC system. Compensatory solutes appeared as peaks with specific retention time and height, and the area under the peak was calculated using the DELTA chromatography system. It was observed that as the incubation time increased, there was a change in the peak size and area under the peak decreased.

Table 7.1: Rate of decomposition (%) \pm SEM of compensatory solutes per minute when 100 mM solutions were incubated at 90°C

Solute	Rate of decomposition(%) / min *
Glycine betaine	0.08 ± 0.01
Dimethylthetin (DMT)	0.05 ± 0.01
Propio betaine (PB)	0.09 ± 0.01
CB-1	0.25 ± 0.02
Deanol betaine (DB)	0.12 ± 0.02
Homodeanol betaine (HDB)	0.37 ± 0.07
Homoglycero betaine (HGB)	0.41 ± 0.05
Diethanol homobetaine (DEHB)	$0.40 \pm 0.03 \%$

* Results from exponential fit of the data from three replicate trials

The area under the peak of a solute sample at different incubation times were compared with its area without incubation and the relative area was calculated. The decrease in the relative area gave a measure of stability of the solute. Plots of decrease in the relative area with the increase in incubation time are as shown in Figure 7.2.

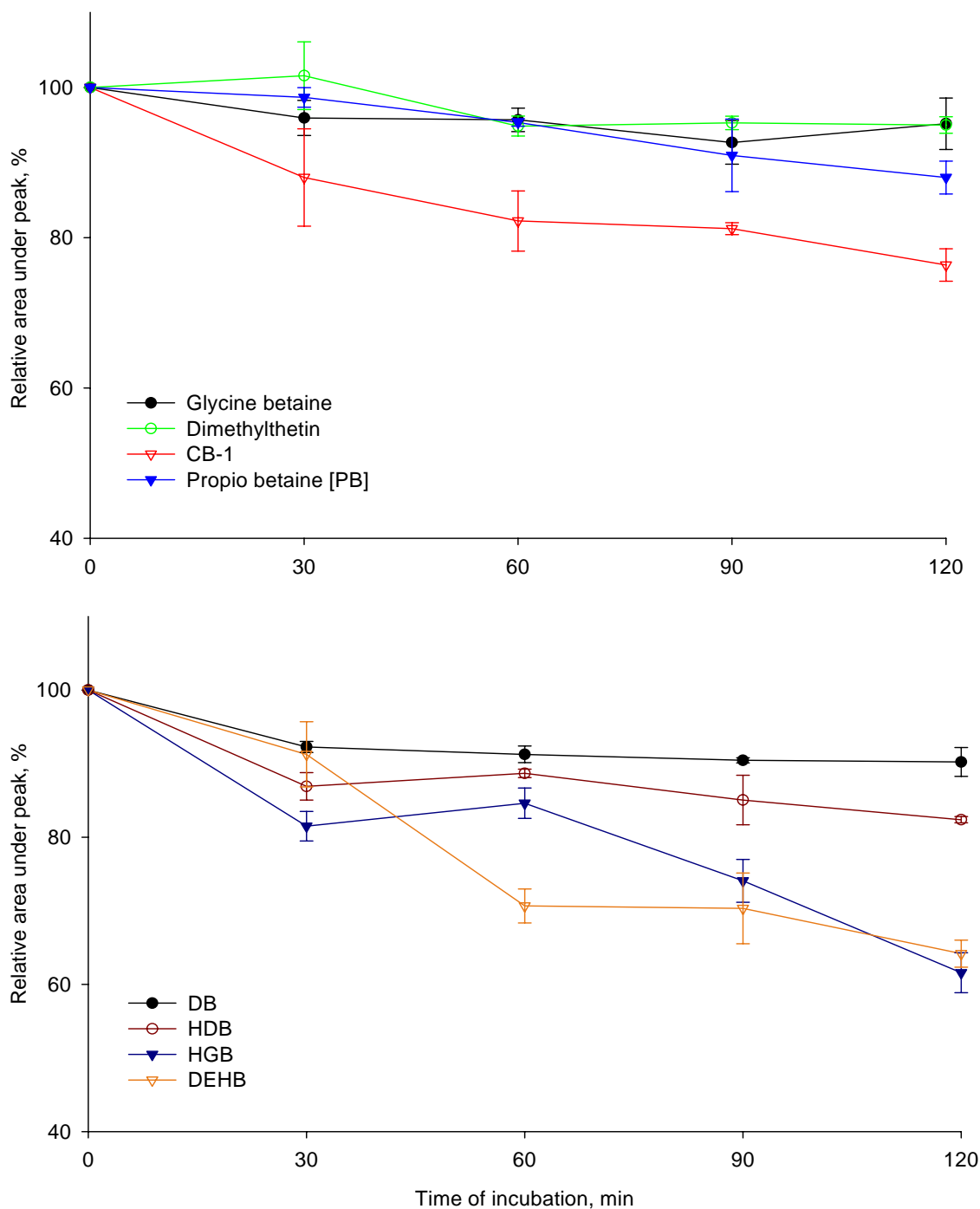


Figure 7.2: Decomposition of 100 mM compensatory solution when incubated at 90°C. The decrease in relative area under the peak with increase in time of incubation represents the stability of the solute. The relative area is relative to the area under the peak for each solute without incubation

From 7.2 and Table 7.1 we observe that GB, propio betaine and DMT seem to be fairly stable compared to DB, HDB, HGB and DEHB. The rate of decomposition is less than 0.1% per minute for GB, propio betaine and DMT. In case of solutes containing –OH groups, their stability decreased with the increase in the size of the molecule. HGB and DEHB decomposed approximately at a rate of 0.4% per minute at 90°C. HGB and DEHB, which have two –OH groups are less stable than DB and HDB with one –OH group. Cyclic betaine, CB-1, is also not very stable as it decomposes at an approximate rate of 0.25% per minute at 90°C.

Stability of sulfobetaines was not studied using this method as the UV detector could not detect sulfonic acid solutes. Attempts were made to use a refractive index (RI) detector instead of an UV detector. However, due to inconsistencies in the results we resorted back to the UV detector.

7.3.2 Density, Viscosity and Hydration Number

Results of density and viscosity measurements are listed in Table 7.2. As expected, dynamic viscosities of sorbitol and trehalose are higher compared to other solutes. Of all the synthetic compensatory solutes, solutes containing two –OH groups in their structure, that is HGB, DEHB and SB-4, have high dynamic viscosities. Dynamic viscosities of HDB and CB-1 are approximately 0.002 Pa.s and are in the higher range as well.

Table 7.2 also lists the apparent hydration numbers of different compensatory solutes calculated assuming spherical molecules. Trehalose has the highest apparent hydration number of 10.7. Synthetic solutes, HGB and DEHB have a hydration number of around 5.0, which is similar to that of sorbitol. GB, DMT, CSB-1 and urea have very low hydration numbers, with CSB-1 and urea having negative hydration numbers. Hydration number of sulfobetaines seemed to increase with the increasing distance between S and N in their structure. This is evident as hydration number of SB-1 is around 1.1 and that of SB-3 is 3.3, which has two extra CH₂'s between its S and N compared to SB-1. Similarly, the apparent hydration numbers of GB is around 0.7 and that of propio betaine is around 1.7, which has an extra CH₂ between its charges compared to GB.

Table 7.2: Density and dynamic viscosities of 23% w/w compensatory solutions. * Apparent hydration number, H , is the number of water molecules associated with each molecule of solute in solution. The uncertainties of H are based on the density and viscosity uncertainty only and do not take into account the spherical molecule assumption.

Solute	Density, kg/m ³	Dynamic Viscosity, Pa.s	Apparent Hydration Number (H) [*]
Sorbitol	1083.2 ± 0.5	2.26 x 10 ⁻³	5.2 ± 0.0
Trehalose	1087.3 ± 0.0	2.11 x 10 ⁻³	10.7 ± 0.1
Glycine betaine	1031.7 ± 0.4	1.70 x 10 ⁻³	0.7 ± 0.0
Propio betaine [PB]	1033.6 ± 0.2	1.83 x 10 ⁻³	1.6 ± 0.0
Deanol betaine [DB]	1050.5 ± 0.5	1.83 x 10 ⁻³	1.2 ± 0.0
Homodeanol betaine [HDB]	1049.1 ± 0.4	2.01 x 10 ⁻³	2.4 ± 0.1
Homoglycerol betaine [HGB]	1056.6 ± 0.5	2.17 x 10 ⁻³	5.3 ± 0.0
Diethanol homobetaine [DEHB]	1057.9 ± 0.5	2.30 x 10 ⁻³	5.0 ± 0.1
CB-1	1052.5 ± 0.3	1.96 x 10 ⁻³	3.4 ± 0.1
SB-1	1065.3 ± 0.2	1.69 x 10 ⁻³	1.0 ± 0.0
SB-2	1061.5 ± 0.6	1.76 x 10 ⁻³	1.6 ± 0.1
SB-3	1049.8 ± 0.4	1.98 x 10 ⁻³	3.3 ± 0.0
SB-4	1024.1 ± 0.3	2.19 x 10 ⁻³	4.3 ± 0.0
CSB-1	1064.1 ± 0.7	1.51 x 10 ⁻³	-0.9 ± 0.1
CSB-2	1050.2 ± 0.6	1.99 x 10 ⁻³	3.2 ± 0.1
Dimethylthetin [DMT]	1054.0 ± 0.6	1.56 x 10 ⁻³	0.3 ± 0.1
Urea	1062.2 ± 0.6	1.26 x 10 ⁻³	-1.1 ± 0.0

The increase in apparent hydration number is also observed in betaine analogues with hydroxyl groups. Hydration number of HGB (with two –OH groups) is around 5.3, which is twice that of HDB with one –OH group. Sulfobetaine, SB-4, with two –OH groups also has a high hydration number of 4.3. Another interesting result is that hydration number of CB-1 is around 3.4 and that of CSB-1 is -0.9, which is different even though both are morpholinium compounds. However, CB-1 is a carboxylic acid analogue and CSB-1 is a sulfobetaine. Cyclic sulfobetaine CSB-2 (piperidium compound) also has a high hydration number of around 3.2.

7.3.3 Theoretical Calculations

The dipole moments and the polarizability of various compensatory solutes calculated at various levels of theory are listed in Table 7.3 and 7.4 respectively.

Table 7.3: Calculated dipole moments (in debye)

Method:	HF	MP2 μ/D	B3LYP
Solute:			
Glycine betaine	11.97	11.18	10.85
Propio betaine	17.91	16.98	16.40
Dimethylthetin	10.16	9.24	8.78
HDB	19.32	12.04	13.09
HGB	19.37	19.17	17.77
SB-2	25.60	25.03	24.35
CB-1	17.61	11.49	10.94
Urea	4.00	3.35	3.49
DMSO	5.00	4.05	3.93

All compensatory solutes have a negatively charged end and a positively charged end. All synthetic compensatory solutes have a large dipole moment, except for DMT. Sulfobetaine SB-2 has the largest dipole moment of all the synthetic solutes. Natural solute glycine betaine has a relatively smaller dipole moment. Dipole moments of urea and DMSO are smaller than other solutes.

Table 7.4: Calculated polarizability

Method:	HF $/\text{\AA}^3$	B3LYP
Solute:		
Glycine betaine	8.71	9.69
Propio betaine	10.21	0.00
Dimethylthetin	8.97	10.09
HDB	12.13	13.15
HGB	13.99	16.05
SB-2	14.60	16.58
CB-1	13.06	14.10
Urea	3.39	3.84
DMSO	6.09	6.30

From Table 7.4, we see that the calculated polarizabilities of synthetic compensatory solutes are high compared to other solutes, except for DMT. HGB and SB-2 have the highest polarizability, and urea and DMSO the lowest.

The structures of number of compensatory solutes studied are as shown in Figures 7.3 A, B and C. The calculations reported are at the same level of theory for all molecular species. The charges are Mulliken population analyses corresponding to the MP2 energy.

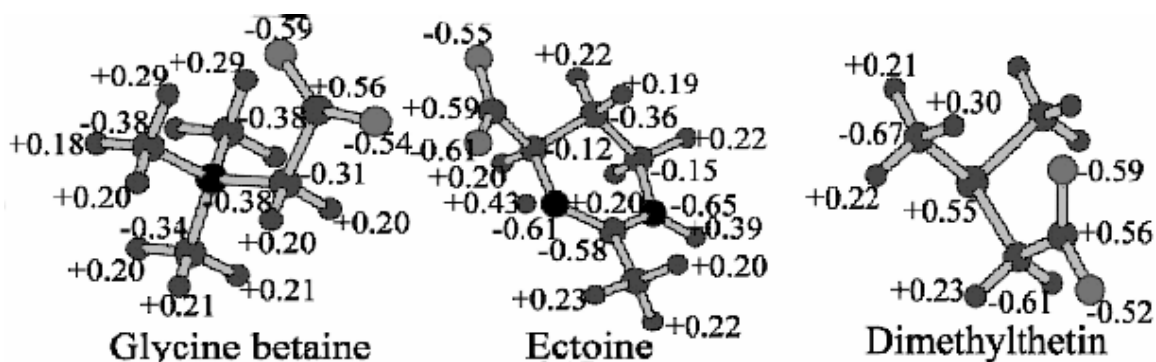


Figure 7.3 A: Structures and Mulliken charges of compensatory solutes (*Lever et al., 2001*)

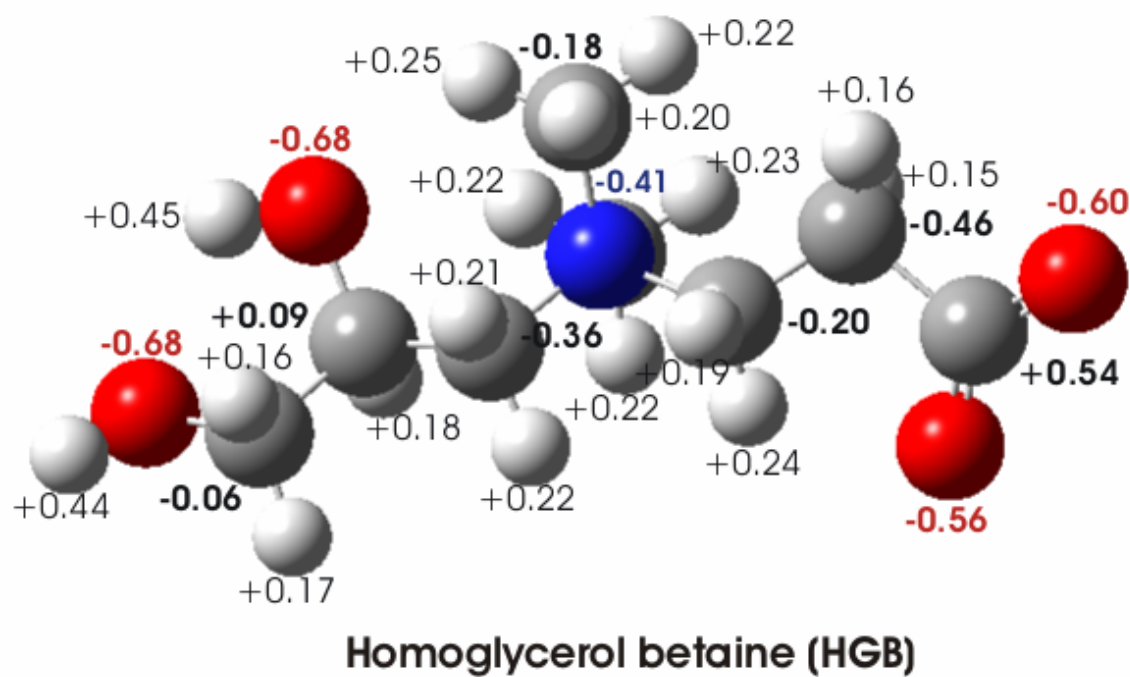
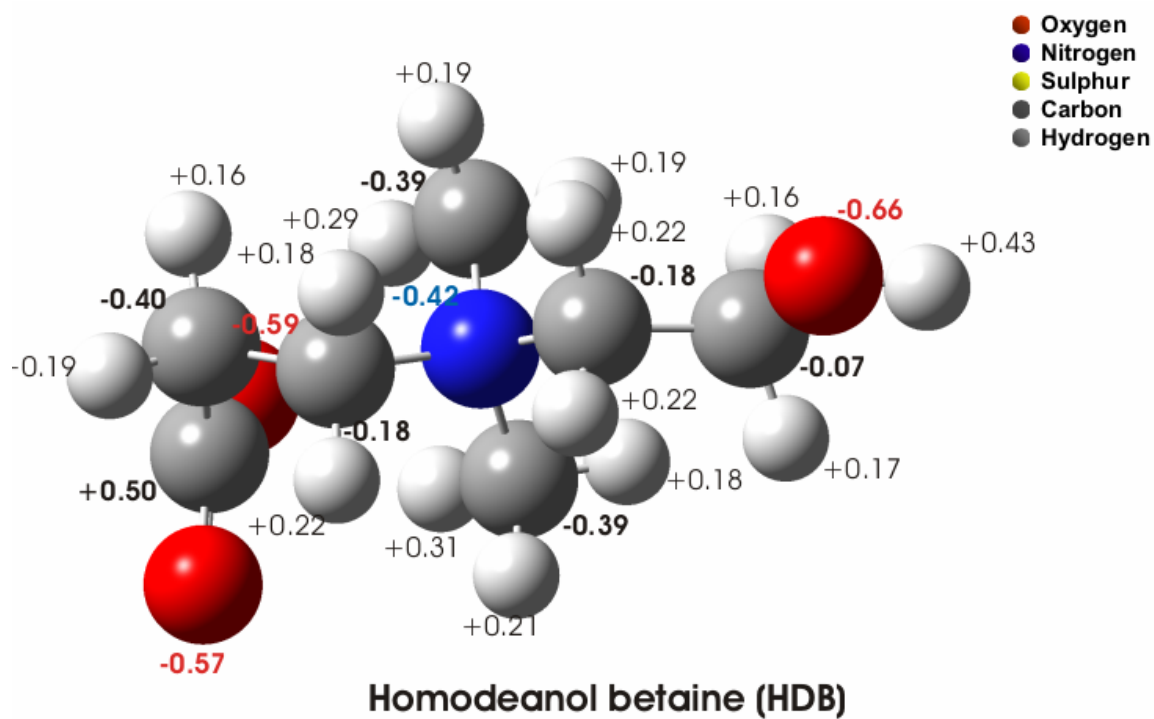


Figure 7.3 B: MP2/ 6-31G structures and Mulliken charges for HDB and HGB

7.4 DISCUSSION

Only a few synthetic solutes (carboxylic acid analogues) were used for the stability experiments as the UV detector could not be used to analyze sulfobetaines (Storer and Lever, 2006). A few trials were carried out using a refractive index detector (Jasco) instead of a UV detector, which allowed us to analyze all the solutes including sulfobetaines. However, results obtained using the RI detector were inconsistent and so it was again replaced by an UV detector and a standard derivatization method (Storer et al., 2006) was followed. The results of stability of sulfobetaines (results not shown) obtained using the RI detector gave us a qualitative indication that sulfobetaines are more stable than the carboxylic acid analogues.

It was shown in Chapter 6 (section 6.3.2) that a number of synthetic solutes such as HDB, HGB and CB-1 that were very good stabilizers of LDH and chymotrypsin against heat stress, failed to protect *Taq* polymerase when it was incubated at 95°C. The reason for this failure is evident from the results of stability experiments. These results clearly show that CB-1, HDB and HGB decompose at a higher rate than the other solutes when heated at 90°C. Consequently, these solutes might have decomposed faster during the incubation of *Taq* polymerase at 95°C for two hours, leading to the formation of starting materials which would have inhibited the activity of the enzyme. This was not the case during experiments with LDH and chymotrypsin as the enzymes were heated with the compensatory solutes for only 10 min and the maximum temperature used was 70°C. Thus, the solutes were able to protect LDH and chymotrypsin but failed with *Taq* polymerase.

Density and viscosity measurements were carried as some of the solutes used in this thesis are new and not much physical data is available for them. The density and viscosity results were further used to calculate the apparent hydration number, H , of the solutes. The apparent hydration numbers of different solutes provide us with a picture of interaction of water molecules with the solute molecules. The apparent hydration numbers indicate the number of water molecules that are associated with a solute

molecule in solution. These numbers might give some insight in to the mechanism of protein stabilization by compensatory solutes.

Results show that the hydration numbers of sorbitol and trehalose are different from those of synthetic betaines. This may be due to the fact that sorbitol is a polyol and trehalose is a disaccharide, and have different structures compared to the synthetic betaines, which have some structural similarities. It is observed that solutes that are good protein stabilizers (e.g. HDB, HGB, CB-1 and SB-3) have higher hydration numbers compared to moderate stabilizers (e.g. GB, DMT and SB-1). This is supported by the negative hydration number of urea, which is a denaturant and known for its protein destabilizing effects. Also observed from the results is that the apparent hydration number of trehalose is almost twice of HGB. However, results of protein stabilization (Chapter 4) showed that HGB is a better stabilizer than trehalose during heat denaturation of enzymes.

Wiggins (1990) published a model describing structural differences between hydration water and bulk water, according to which two layers of water are found around a protein; dense (or weakly bonded) water in the hydration shell of proteins and less dense water (or structured water) in bulk. Based on this theory, Galinski (1993) suggested that different solutes will show preferences to one or both the water layers: small highly charged molecules preferentially dissolve in hydration water, whereas large molecules of low charge density are better dissolved in bulk water. Galinski (1993) determined the hydration numbers of natural compatible solutes (glycine betaine and ectoine) using near-infrared spectroscopy, and based on these hydration numbers they concluded that compatible solutes have relatively large hydration shells. Thus, compatible solutes because of their large hydration shells prefer to dissolve in the bulk water, which is more structured form of water. Our results of apparent hydration numbers of synthetic compensatory solutes support these suggestions by Galinski (1993). However, our results can only be considered as an estimate of hydration numbers as we have assumed all solute molecules to be spherical in our calculations. From the results, it is also observed that synthetic solutes that are good protein stabilizers have higher apparent hydration numbers compared to the rest. This shows that the solutes prefer the bulk water (less

dense water) resulting in the preferential exclusion of compensatory solutes from the hydration shell of the proteins.

In a study by Turner (2000), they showed that under low water conditions the stability of enzymes increases and makes them possible to perform biotransformations at higher temperatures. They suggested that at low water activity enzymes are catalytically active at higher temperatures. The apparent hydration numbers of solutes which protect enzymes against heat denaturation are high (e.g. HGB, HDB etc), which suggests that the solutes attract more water molecules towards them in solution reducing the amount of water available for the protein, which in turn might be helping the enzymes to maintain their catalytic activity at higher temperatures.

These are just speculative arguments in an effort to understand more about how these solutes stabilize the proteins. These arguments are not based on clear and precise results as we assumed all solute particles to be spherical while calculating the apparent hydration numbers. Also, it has been stated that these calculations have empirical validity only, and that no precise meaning should be attached to the apparent hydration numbers (Edward, 1957). In an investigation using quantum mechanical calculations and QM/MM molecular dynamic simulation (Sironi et al., 2001), the effect of glycine betaine on structure and dynamics of the surrounding water was compared with those of trehalose. They calculated the of average number of water molecules residing within 3.5 Å of the solute atoms (first-shell waters) and those that are hydrogen bonded to the solutes (H-B waters), and showed that glycine betaine and trehalose have 7.0 and 18.9 water molecules respectively in their H-B waters. In case of trehalose, their values are comparable to our calculated apparent hydration number. However, in case of glycine betaine, it is evident that the apparent hydration number by our calculations seems to be less compared to their calculations.

An attempt was made to determine if there was a correlation between the hydration numbers of solutes and the relative activity of enzymes during heat denaturation and freeze-drying. Figure 7.4 shows the plot of apparent hydration number of solutes versus the relative activity of LDH after freeze drying in their presence.

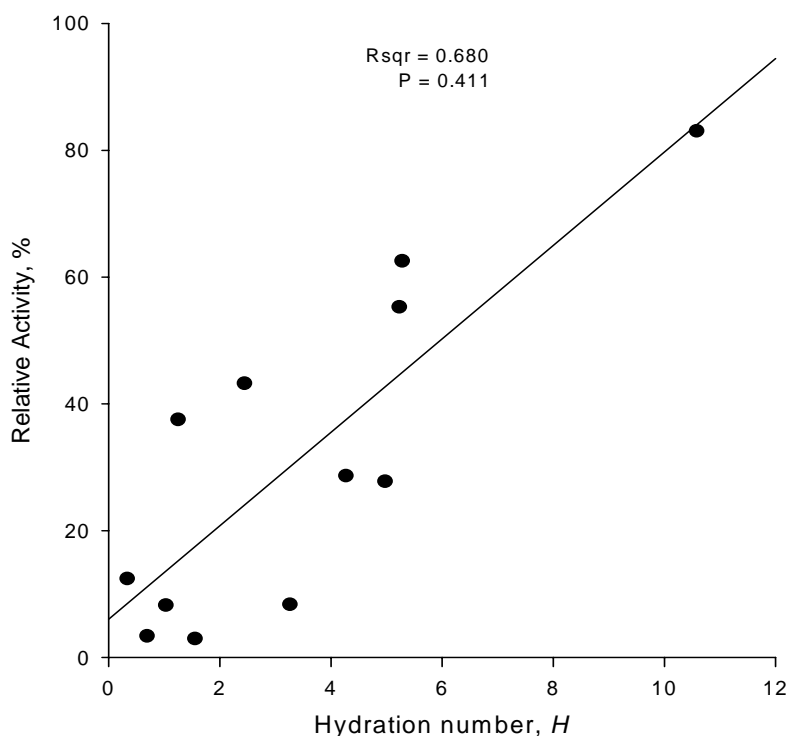


Figure 7.4: Relation between hydration numbers (H) of compensatory solutes and relative activity of LDH after freeze-drying in the presence of different 1 M compensatory solutions.

Though Figure 7.4 does not show a perfect correlation, it gives an indication that the increase in the hydration number of solutes increases its ability to protect LDH against freeze-drying. This shows that the solute molecules which have more water molecules in their hydration envelope tend to give better water substitution to enzymes during the dehydration stage of freeze-drying. To determine if there was a relation between protection against heat denaturation of enzymes and apparent hydration numbers of solutes, we plotted the change in T_{D-50} ($^{\circ}\text{C}$) of LDH and chymotrypsin in the presence of different solutes against hydration numbers (Figure 7.5). However, no apparent relation could be observed. Changes in T_{D-50} temperatures versus dynamic viscosity of 1 M compensatory solutions was also plotted to determine if any relation existed between the viscosity of the solution and the observed stabilization effect (Figure 7.6). Again, no relation could be deduced from this plot.

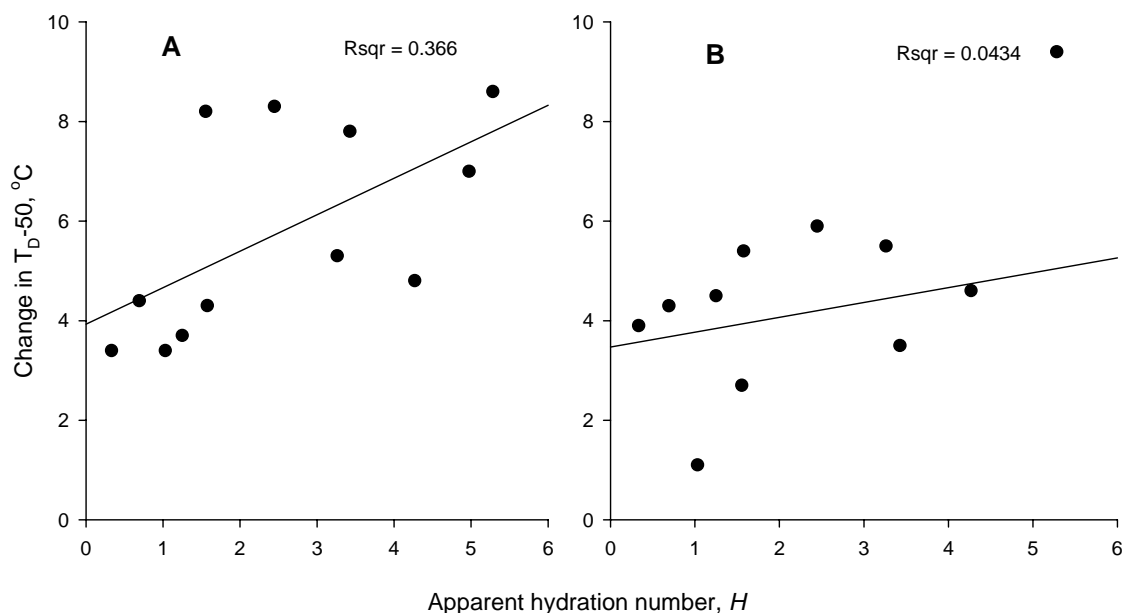


Figure 7.5: Relation between apparent hydration number (H) and change in T_{D-50} (change in temperature at which 50% of enzyme activity is lost, Table 4.1, Chapter 4) in the presence of different compensatory solutes (A) LDH (B) Chymotrypsin.

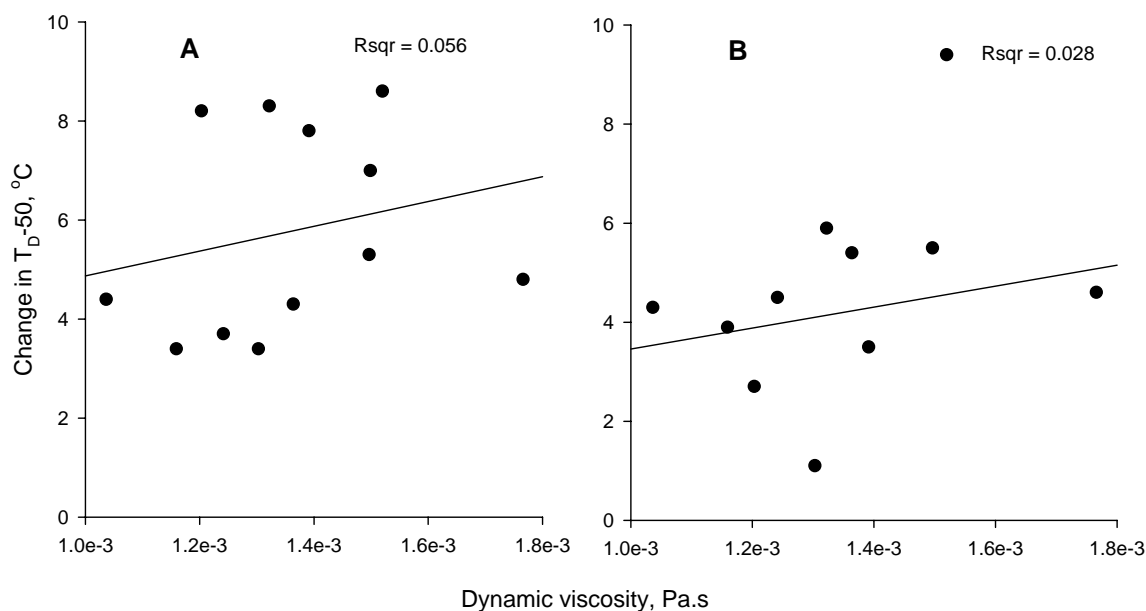


Figure 7.6: Relation between dynamic viscosities of 1 M compensatory solutions and changes in T_{D-50} (change in temperature at which 50% of enzyme activity is lost, Table 4.1, Chapter 4) (A) LDH (B) Chymotrypsin.

The structures of compensatory solutes in Figure 7.3 A, B and C were determined at MP2/6-31G level of theory. The structures are “gas-phase” structures, but should be a

good approximation to structure in solution. It was possible to calculate the dipole moment and polarizability of solutes from the electronic wave functions. It is evident from Table 7.3 and 7.4 that synthetic solutes HDB, HGB, SB-2 and CB-1, which are good protein stabilizers, have large dipole moment and high polarizability compared to other compensatory solutes. The large dipole moments could be responsible for significant interactions with other molecules and water molecules. They could also modify the water structure in solutions. The large dipole moment and polarizability of HDB and HGB correlate well with the calculated apparent hydration numbers, which suggests that these solutes strongly interact with water molecules. These results could also imply that the solutes have higher affinity towards the bulk water and get preferentially excluded from the protein surface, which results in good protein stabilization. All these results further support the preferential exclusion/ hydration model.

7.5 REFERENCES

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Chapter 8 - Discussion and Conclusions

8.1 DISCUSSION

The discussion about specific applications of synthetic compensatory solutes is contained within the relevant chapters. In this general discussion an attempt is made to consider the effects of compensatory solutes in various applications and to determine if there are any general effects observed within any particular group of compensatory solutes.

The syntheses and different biomedical applications of a range of novel compensatory solutes have been investigated in this thesis. The majority of compensatory solutes were easy to synthesize and a few of them were inexpensive compared to their natural counterparts. Though the purity and the yield of a few solutes are a matter of concern, we believe that designing an industrial scale process of syntheses would result in better products. As the solutes were synthesized during different stages of the three year project, a few solutes have not been used in some experiments described in the thesis.

Thermostable enzymes have allowed for the development of techniques such as PCR. However, the majority of the thermostable enzymes are obtained from thermophilic organisms, and their isolation and purification makes them expensive. To expand the availability of these thermostable enzymes, scientists have explored novel methods such as addition of co-solvents and structure stabilizing solutes in order to thermostabilize enzymes that are thermolabile. This work presents the thermostabilization of different model proteins by synthetic compensatory solutes. As a result, it enabled the identification of a number of novel thermostabilizers of enzymes. In the presence of these stabilizers, the enzymes were able to maintain their catalytic activity even at temperatures where the enzyme, in the absence of solutes, would have been rendered inactive. It was also shown that the observed stabilization effects were not just on the catalytic activity of the enzyme but also on the structural conformation.

Thermostabilization and thermoactivation of enzymes has the potential to pave the way for various new applications in a wide range of fields. In fact, the combination of thermostabilization and increasing the enzyme's catalytic activity may be very helpful in reducing the quantity of enzyme used in many industrial and laboratory scale applications. Furthermore, this increase in activity would increase the speed of a given reaction and thus reduce the time required.

Table 8.1 summarizes the effects of various compensatory solutes in some of the applications investigated in this thesis. It is evident from the table that trehalose lives up to its title as a “universal stabilizer” as it is consistent in its stabilization effect on various proteins and stress factors. Sorbitol shows a similar trend though its stabilization effect is not as good as trehalose. Table 8.1 also shows that synthetic solutes are good stabilizers of proteins. During stabilization against heat, synthetic solutes such as HDB and HGB show superior performance than the other compensatory solutes, including trehalose. However, during stabilization against low temperatures (e.g. freeze-drying), it is the natural solutes such as trehalose which exhibit outstanding stabilization effect. But, synthetic solutes do not fail completely here. Solute such as HGB and HDB, which show considerable stabilization during freeze-drying, could be a cheaper option to trehalose when considered on a gram basis. They can even be used along with trehalose (mixtures) which could bring down the cost of freeze-dried products.

One common feature that is observed from the study of stabilization of proteins is that compensatory solutes with OH groups are good stabilizers when compared to other solutes. Trehalose, sorbitol, HGB, HDB, DB and sulfobetaines have one or more OH groups and are good protein stabilizers. This raises the question whether OH/hydroxyl group is a pre-requisite for a solute to be a good protein stabilizer. Though exact conclusions cannot be drawn on this, the results of this work suggest that this is true. The mechanisms of protein stabilization that have been proposed in the past (detailed in Chapter 2) point out that solutes which are good protein stabilizers are good water structure formers (kosmotropes) and are excluded from the immediate vicinity of the protein surface. Results presented in Chapter 7 of this thesis tell the same story about

synthetic solutes. The good protein stabilizers such as HDB and HGB have a bigger apparent hydration number, high dipole moment and polarizability. These results suggest that stabilizing solutes tend to interact more with water and thus have more affinity towards the bulk water than the protein surface, which in turn suggest preferential exclusion of the solutes from the surface of the protein. Thus, our results seem to agree well and support the preferential exclusion model of protein stabilization (2.2.1, Chapter 2).

Another interesting observation that we can draw from Table 8.1 is that during freeze-drying only solutes with OH groups are able to confer protection to the enzymes and the solutes without OH groups fail completely. This supports the mechanism and evidence provided by Carpenter and co-workers (2.2.4.2, Chapter 2), who suggested that solutes are able to protect proteins during freeze-drying only because they hydrogen-bond to the dried protein, acting as water substitutes. This is well supported by our results which show that GB, propio betaine, CB-1 and DMT due to their lack of hydroxyl group fail to protect LDH during freeze-drying.

Though the results presented in this work tend to support some of the previously proposed protein stabilization mechanisms, we believe that there is no any one particular mechanism by which the stabilization of proteins can be explained. Previous workers have considered only a few natural stabilizers for their studies of mechanism of protein stabilization by co-solvents. These studies if carried out again considering the novel synthetic solutes presented in this thesis, would throw more insight into the stabilization mechanisms. Also, previously two types of solutes were considered for protein stabilization studies; zwitterionic stabilizers that had high dipole moments (e.g. amino acids, TMAO, GB), and polyols which formed hydrogen bonds with both water and proteins due to the presence of hydroxyl groups. In this work, we have investigated solutes that are both zwitterionic and have hydroxyl groups. In this way we have tried to combine the two different effects. Similar work was carried out by Lippert and Galinski (1992) with their study of enzyme stabilization using ectoine and hydroxyectoine, two natural compatible solutes.

Table 8.1: Summary of effects of various compensatory solutes in different applications investigated in this thesis. (Stabilization of Proteins: ✓ - stabilizer and ✗ - no effect, DNA melting: ‘*Increase*’ – stabilizes DNA and ‘*Decrease*’ – reduces DNA melting temperature, PCR Enhancer: ✓ - potential enhancer and ✗ - no enhancing effect) (Note: *ND* – Data not available)

Solute	Stabilization against heat					Freeze-thawing	Freeze-drying		Effect on DNA melting temperature	PCR Enhancer
	LDH	Chymotrypsin	CBG	Taq Polymerase	Lipase	LDH	LDH	Lipase		
<u>Natural Solutes</u>										
GB	✓	✓	✓	✓	✗	✓	✗	✗	<i>Decrease</i>	✓
Trehalose	✓	✓	✓	✓	✓	✓	✓	✓	<i>Decrease</i>	✓
Sorbitol	✓	✓	✓	✓	✓	✓	✓	✓	<i>Decrease</i>	<i>ND</i>
<u>Synthetic Solutes</u>										
Propio betaine	✓	✓	✓	✗	<i>ND</i>	✓	✗	<i>ND</i>	<i>Increase</i>	<i>ND</i>
DB	✓	✓	✓	✓	<i>ND</i>	✓	✓	<i>ND</i>	<i>Increase</i>	<i>ND</i>
HDB	✓	✓	✓	✗	✓	✓	✓	✓	<i>Increase</i>	<i>ND</i>
HGB	✓	✓	✓	✗	<i>ND</i>	✓	✓	<i>ND</i>	<i>Increase</i>	<i>ND</i>
DEHB	✓	✗	✓	✗	<i>ND</i>	✓	✓	<i>ND</i>	<i>ND</i>	<i>ND</i>
CB-1	✓	✓	✗	✗	<i>ND</i>	✓	✗	<i>ND</i>	<i>Decrease</i>	<i>ND</i>
DMT	✓	✓	✗	✓	✗	✓	✗	✓	<i>ND</i>	
<u>Sulfobetaines</u>										
SB-1	✓	✗	✗	✓	<i>ND</i>	✓	✗	<i>ND</i>	<i>ND</i>	<i>ND</i>
SB-2	✓	✓	✗	✓	<i>ND</i>	✓	✓	<i>ND</i>	<i>Decrease</i>	✓
SB-3	✓	✓	✓	✓	<i>ND</i>	✓	✗	<i>ND</i>	<i>Increase</i>	<i>ND</i>
SB-4	✓	✓	✗	✗	<i>ND</i>	✓	✓	<i>ND</i>	<i>Decrease</i>	<i>ND</i>
CSB-1	<i>ND</i>	<i>ND</i>	<i>ND</i>	✓	<i>ND</i>	<i>ND</i>	<i>ND</i>	<i>ND</i>	<i>Decrease</i>	✓
CSB-2	<i>ND</i>	<i>ND</i>	<i>ND</i>	✗	<i>ND</i>	<i>ND</i>	<i>ND</i>	<i>ND</i>	<i>Decrease</i>	<i>ND</i>

It is suggested that, in order to stabilize a protein against an external stress, it is better to have a range of stabilizers to choose from (as presented in this work) and select the best stabilizer for that particular protein and the stress factor. Although protein stabilization applications would require a case by case selection of the synthetic solutes (as their effect may be enzyme specific or stress specific), the simplicity of stabilization of these synthetic solutes has a great potential to improve the cost and time involved in a wide range of enzymatic applications, biochemical reactions, diagnostics and industrial fields. Also, this kind of stabilization would increase the overall efficiency, and thus allow yields unexpected under standard conditions.

Apart from investigating the important application of the synthetic compensatory solutes, that is protein stabilization, this thesis also examined applications such as their effect on DNA melting temperature and PCR enhancers. Table 8.1 summarizes the effect of various compensatory solutes on the DNA melting temperature. The mechanism by which compensatory solutes destabilize the DNA double helix is not well understood. A number of mechanisms have been proposed based on the results of a few natural compensatory solutes. This thesis presents a new range of synthetic compensatory solutes which have different effects on DNA melting temperatures.

It is observed that a slight change in the structure of the solute (e.g. distance between the charges), changes the effect of the solute on the DNA completely. Comparing the effect of GB and propio betaine on the DNA melting temperature, where GB reduces the melting temperature and propio betaine increases it, we see that the only structural difference between these two is that propio betaine has an extra CH_2 between its charges (see Appendix A for the complete list of structures of compensatory solutes). Similarly, opposite effects are observed with SB-2 and SB-3, where SB-3 increases and SB-2 decreases the DNA melting temperature. This is a rather more interesting result as all other sulfobetaines considered in this work decrease the melting temperature except for SB-3.

Further interesting results on DNA melting temperature were obtained with solutes with OH groups. Results showed that trehalose and sorbitol stabilize the proteins and destabilize the DNA, whereas, synthetic solutes DB, HDB, HGB and DEHB stabilize both proteins and DNA. However, sulfobetaines SB-1, SB-2 and SB-4, though they have OH groups in their structures, destabilize the DNA double helix. Comparing HGB and SB-4, it is observed that both are synthetic solutes and both have two OH groups, but they have completely opposite effects on the DNA melting temperature, that is HGB increases and SB-4 decreases the melting temperature. These interesting properties of synthetic compensatory solutes have provided a platform for better understanding of the mechanism by which compensatory solutes interact with DNA and other macromolecules. Based on observed stabilization of DNA double strand by solutes such as HDB and HGB, we believe that a few synthetic compensatory solutes could find potential applications in the area of DNA storage, as long-term storage of DNA is required for a number of genetic studies (Madisen, 1987).

This work has explored a few potential applications of synthetic compensatory solutes. However, various other applications can also be investigated in a number of industries such as,

- Diagnostic reagents
- Cell stabilization
- Animal products and leather industry.
- Pharmaceutical industry
- Cosmetics
- Food industry

Synthetic compensatory solutes could be expected to function in many of these applications, though they have not been specifically tested for them. Many reagents that contain enzymes are usually supplied with stabilizing substances such as glycerol. These usual stabilizing substances can be replaced by synthetic compensatory solutes which are inexpensive and have better stabilizing properties. A number of commercial PCR

reagents contain GB and DMSO as enhancer solutions, which reduce the melting temperature of double-stranded DNA and also protect the enzyme against high temperatures. Results presented in this thesis show that synthetic solutes are better than GB in reducing the DNA melting temperatures. Hence, synthetic solutes may have a potential to replace GB in PCR enhancer solutions.

Bacteria are used industrially in processes like organic chemical transformations. Cultures of the bacteria can be stored dried or frozen with suitable stabilizing additives. Other mammalian cell types have also been preserved using trehalose and other solutes (Brumfiel, 2004). These can be some of the other potential applications of our solutes. There are also several aspects of pharmaceutical industry such as production of vaccines and other protein based pharmaceuticals where our solutes have market potential. However, if synthetic solutes are to be used internally in humans, then a number of toxicity tests have to be carried out, and at this stage we don't have any data regarding them.

The stabilizing solutes GB and ectoine are being used in cosmetic preparations as active ingredients in skin moisturizers and anti-wrinkle creams (<http://www.merck.com>). We have shown from our results that some of our solutes are more effective than GB and ectoine in stabilizing proteins and are less expensive. We feel that our synthetic solutes have a good commercial potential in the cosmetic industry. Furthermore, less stringent trials are needed to prove that the solutes are safe to be used for application on skin compared to their applications in the pharmaceutical or food industries.

8.2 CONCLUSIONS

8.2.1 Syntheses of compensatory solutes

A range of novel compensatory solutes were synthesized in the laboratory. The majority of the solutes were easy to synthesize and the yield was usually more than 75%. Some of the solutes can be synthesized from inexpensive starting materials and the cost of the

solute is cheaper compared to the natural solutes. A few of the solutes, especially HGB and sulfobetaines, are more expensive to synthesize due to the high price of their starting materials. However, the cost of these expensive solutes could reduce if a large-scale process for their synthesis is designed.

8.2.2 Application of synthetic compensatory solutes as protein stabilizers

This thesis has showed that synthetic compensatory solutes have a potential application as protein stabilizers. Based on the results using a few model proteins, it was shown that synthetic solutes stabilize proteins against the denaturing effects of high and low temperatures. It was also proved that the observed stabilization effect is not just on the catalytic activity of the enzyme, but also on its structural conformation. Based on these results we also conclude that while selecting a suitable stabilizer for a particular protein, it is always better to have a range of stabilizers ready as the stabilization effect of solutes may vary from protein to protein. Thus, we have successfully added a number of inexpensive solutes to the available range of protein stabilizers.

8.2.3 Medical application of betaine analogues

The application of DMT, a synthetic betaine analogue, as a therapeutic agent was not successful. However, from the study we were able to generate a large-animal CAPD model, which to our knowledge is the first attempt. A sheep CAPD model has several advantages over a small animal model and will help researchers to study the same animal repeatedly in both control and uremic conditions. It was also shown that addition of GB in dialysis fluid for chronic renal failure patients can be a viable option as GB was not toxic to sheep when administered via dialysis fluid. This is a very helpful result as long-term oral supplementation with GB in chronic renal failure patients for lowering homocysteine concentration has been problematic.

8.2.4 Synthetic compensatory solutes for reducing DNA melting temperatures and their application as PCR enhancers

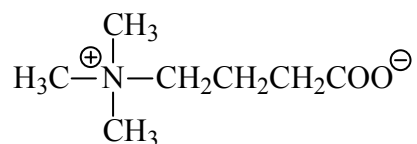
A few of the synthetic compensatory solutes were able to reduce the melting temperatures of DNA better than most of the well known solutes such as GB, trehalose and sorbitol. GB is being used as an active ingredient in a number of PCR enhancers that are being marketed by commercial suppliers. Synthetic solutes CSB-1 and SB-2 were shown to have the potential to replace GC-Rich Resolution Solution supplied by Roche in a Fragile-X PCR assay. Considering *Taq* Polymerase as another model enzyme for the stabilization studies, it was also shown that a few synthetic compensatory solutes are able to protect even a thermophilic enzyme (*Taq* polymerase) against high temperatures.

8.2.5 Physical data for synthetic compensatory solutes

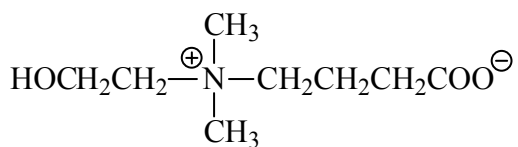
As most of the solutes synthesized in this thesis are new, information has been provided about their stability, density and viscosity. In an attempt to understand more about the mechanisms of protein stabilization, the apparent hydration numbers, dipole moment and polarizability of synthetic compensatory solutes were calculated. Results presented in this thesis seem to agree with the preferential exclusion mechanism of protein stabilization.

8.3 RECOMMENDATIONS FOR FUTURE WORK

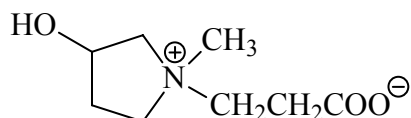
Though only a few of synthetic solutes and their application in a few areas were studied, results of this work have raised the interest for synthesizing more solutes that might be inexpensive and have wider range of applications. Listed below are a number of synthetic compensatory solutes and their possible starting materials that could be synthesized for any future work in this area.



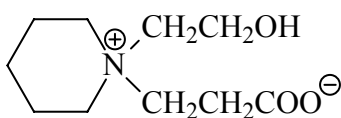
Synthetic Solute 1: Trimethylamine + bromobutyric acid



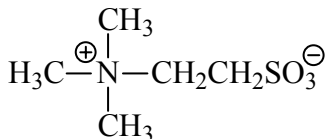
Synthetic Solute 2: Deanol + bromobutyric acid



Synthetic Solute 3: 1-methyl-3-pyrrolidinol + acrylic acid



Synthetic Solute 4: 1-piperidine ethanol + acrylic acid



Synthetic Solute 5: Trimethylamine + vinylsulfonic acid

Other recommended works includes:

- The stabilization effect of synthetic compensatory solutes should be studied with more model proteins and with different stress factors (e.g. urea denaturation). This would give a clear picture whether the observed stabilization effect by solutes is protein or stress specific.
- As more number of novel protein stabilizers is available now, studies to understand the mechanism of protein stabilization can be carried out. Near-infrared spectroscopic studies with the novel compensatory solutes could add to the understanding of their effects on proteins during freeze-drying.

- The circular dichroism (CD) spectra and differential scanning calorimetric (DSC) studies of DNA in the presence of different compensatory solutes could be carried out to study the DNA conformational changes in the presence of different solutes.
- The effectiveness of novel synthetic solutes as PCR enhancers needs to be examined in more GC-rich PCR assays. PCR assays with different denaturing and annealing temperatures must be carried out to identify more PCR enhancers.
- Synthesizing more inexpensive solutes that are similar to presently known PCR enhancers would be of great commercial interest.

8.4 References

Brumfiel, G. (2004) “Cell biology: Just add water”, *Nature*, 428, 14-15.

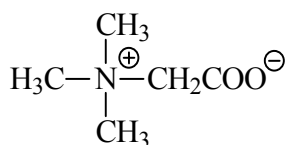
<http://www.merck.de/servlet/PB/menu/1284960/index.html> [Online accessed, 15 April 2006].

Lippert, M. and Galinski, E.A. (1992) “Enzyme stabilization by ectoine-type compatible solutes: protection against heating, freezing and drying”, *Applied Microbiology and Biotechnology*, 37, 61-65.

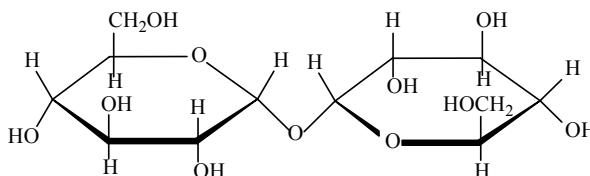
Madisen, L., Hoar, D.I., Holroyd, C.D., Crisp, M. and Hodes, M.E. (1987) “DNA banking: the effects of storage of blood and isolated DNA on the integrity of DNA”, *American Journal of Medical Genetics*, 27(2), 379-390.

Appendix A - Structures of Compensatory Solutes

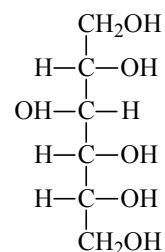
NATURAL COMPENSATORY SOLUTES



Glycine betaine [GB]

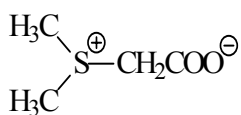


Trehalose

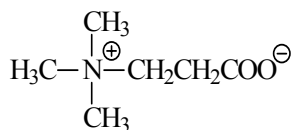


Sorbitol

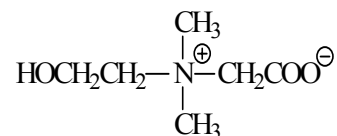
SYNTHETIC COMPENSATORY SOLUTES



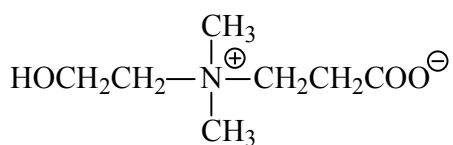
Dimethylthetin [DMT]



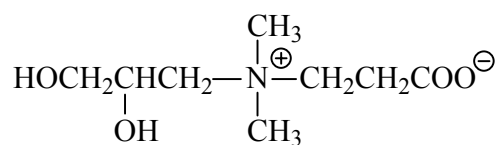
Propio betaine [PB]



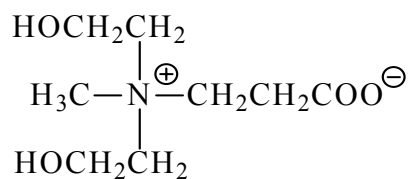
Deanol betaine [DB]



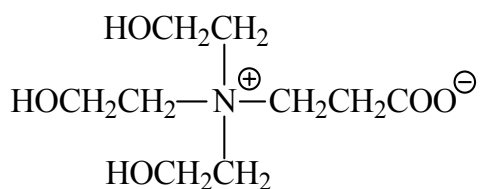
Homodeanol betaine [HDB]



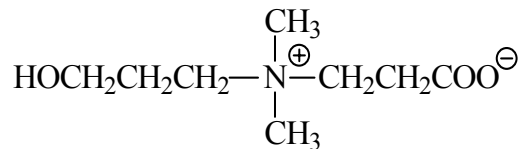
Homoglycerol betaine [HGB]



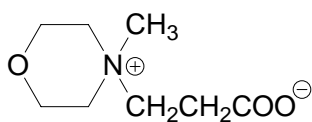
Diethanol homobetaine [DEHB]



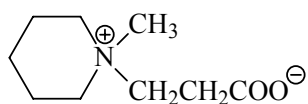
Triethanol homobetaine [TEHB]



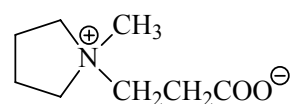
Hydroxypropyl homobetaine [HPHB]

CYCLIC BETAINES

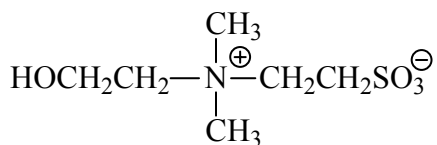
CB-1



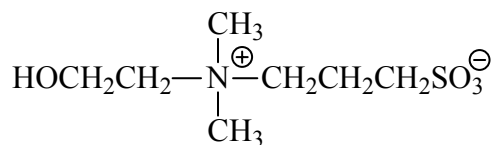
CB-2



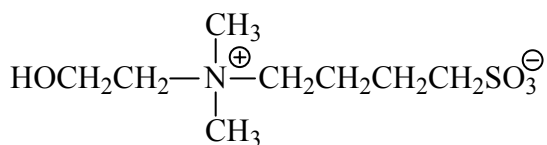
CB-3

SULFO BETAINES

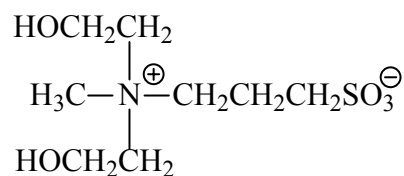
SB-1



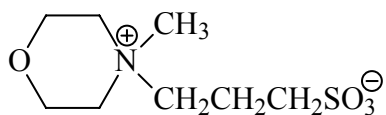
SB-2



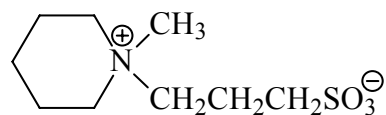
SB-3



SB-4

CYCLIC-SULFO BETAINES

CSB-1



CSB-2